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14. ABSTRACT

The p75 neurotrophin receptor (p75NTR) is a tumor suppressor in the prostate whose expression decreases as prostate cancer progresses. The purpose of this project is to investigate the role of p75NTR in the observed anticancer activity of aryl propionic acids in the prostate. I have found that treatment of prostate cancer cells with the aryl propionic acids R-flurbiprofen and ibuprofen induces reexpression of p75NTR, decreases cell survival, and increases apoptosis. In addition, p75NTR was the only apoptosis inducing death receptor that was significantly upregulated by treatment. Transfection of two different dominant negative forms of p75NTR or p75NTR siRNA before treatment partially rescued the cells from decreased cell survival. Therefore, induction of p75NTR by R-flurbiprofen and ibuprofen is at least partially causal of the observed decreased survival. Investigation into the mechanism of p75NTR induction by R-flurbiprofen and ibuprofen revealed a strong correlation between increased p75NTR protein level and increased p75NTR mRNA level. In addition, the increase in mRNA appears to be the result of increased mRNA stability. Finally, induction of p75NTR seems to be dependent on the p38 MAPK pathway, which is involved in regulating mRNA stability of a subset of transcripts.

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Introduction

The National Cancer Institute estimates there will be 218,890 new cases of prostate cancer and 27,050 deaths from prostate cancer in the United States this year (1). This makes prostate cancer the second most commonly diagnosed cancer type behind nonmelanoma skin cancer, and the second leading cause of cancer related death among men behind lung cancer (1). When detected early at the organ-confined stage, prostate cancer is often curable by surgical removal of the prostate or radiation therapy. However, when prostate cancer has spread outside of the prostate or has recurred following surgery or radiation therapy, current treatment involves some form of androgen deprivation therapy, and essentially always results in incurable hormone-refractory prostate cancer (HRPC). Once the cancer has reached this advanced stage, treatment options are limited and relatively unsuccessful. Docetaxel is the most successful drug to date and extends the life of the patient an average of two to three months. Therefore, there is a great need for improved treatment of advanced HRPC.

Nonsteroidal anti-inflammatory drugs (NSAIDs) demonstrate promise in both prevention and treatment of prostate cancer. They are traditionally used to treat inflammation by inhibiting cyclooxygenase (COX) activity. Increased COX-2 expression is believed to contribute to tumorigenesis through several mechanisms including stimulation of growth, promotion of angiogenesis, increased inflammation, increased invasion and migration, immune suppression, and inhibition of apoptosis (2). However the role of COX-2 in prostate cancer is controversial. Although, reports of COX-2 expression in prostate cancer vary, long term NSAID use is associated with decreased prostate cancer risk, and several COX inhibitors consistently induce apoptosis in prostate cancer cells regardless of COX-2 expression (3-7). Importantly, the efficacy of NSAIDs in inhibiting prostate cancer cell growth has been demonstrated in androgen nonresponsive cells such as PC-3 and DU-145, indicating potential for these drugs in the treatment of advanced prostate cancer (7-8). This is an exciting possibility given the limited options available for patients with HRPC. In particular, the aryl propionic acid class of NSAIDs, or profens, have repeatedly demonstrated anticancer activity in the prostate. This class of NSAIDs includes flurbiprofen, ibuprofen, naproxen, and ketoprofen among others. Long term ibuprofen use is associated with a decreased risk of prostate cancer (9-10). Treatment with the enantiomer R-flurbiprofen, which lacks COX inhibitory activity, was able to inhibit progression of prostate cancer in the TRAMP mouse (11). In addition, ibuprofen treatment decreased survival and induced apoptosis of DU-145 and LNCaP cells (7). This result was also observed in LNCaP cells treated with naproxen (7).

The p75 neurotrophin receptor ($p75^{NTR}$) is an important player in the development of prostate cancer. It is a member of the tumor necrosis factor receptor superfamily (TNFR), and acts as a tumor suppressor in the prostate by inducing apoptosis and suppressing growth through its intracellular death domain (12-13). However, expression of $p75^{NTR}$ is decreased as prostate cancer progresses and is minimal in established advanced prostate cancer cell lines such as PC-3, DU-145, and LNCaP (14-15). Exogenous reexpression of $p75^{NTR}$ in prostate cancer cells resulted in decreased proliferation and increased apoptosis that was dependent upon the death domain of $p75^{NTR}$ (16). This indicates that drugs which induce reexpression of $p75^{NTR}$ in prostate cancer cells may have therapeutic potential. Interestingly, treatment of DU-145 prostate cancer cells with ibuprofen resulted in increased $p75^{NTR}$ expression (17). Therefore, it seems possible that induction of $p75^{NTR}$ may be causal of the observed anticancer activity of aryl propionic acids in the prostate.

Body

Task 1: I examined the effect of selected aryl propionic acid NSAIDs and structurally related compounds on the decreased survival of prostate cancer cell lines PC-3, DU-145, and LNCaP by induction of the $p75^{NTR}$ protein. The $p75^{NTR}$ has been shown to function as a tumor suppressor in the prostate by virtue of its intracellular death domain that can initiate apoptosis and inhibit growth. The most efficacious compounds for induction of $p75^{NTR}$ and decreased survival, in rank-order, were R-flurbiprofen, ibuprofen, oxaprozin, fenoprofen, naproxen, and ketoprofen. Since R-flurbiprofen and ibuprofen exhibited the greatest efficacy, I examined their dose-dependent specificity of induction for $p75^{NTR}$ relative to other members of the death receptor family. Whereas treatment with R-flurbiprofen or ibuprofen resulted in a massive induction of $p75^{NTR}$ protein levels, the expression of Fas, $p55^{TNFR}$, DR3, DR4, DR5, and DR6 remained largely unchanged. Moreover, transfection of either cell line prior to R-flurbiprofen or ibuprofen treatment with a dominant negative form of $p75^{NTR}$ to antagonize $p75^{NTR}$ activity or $p75^{NTR}$ siRNA to prevent $p75^{NTR}$ protein expression rescued both cell lines from decreased survival. Hence, R-flurbiprofen and ibuprofen selectively induce $p75^{NTR}$-dependent decreased survival of prostate cancer cells independently of COX inhibition. Previously our lab demonstrated that loss of $p75^{NTR}$ expression in prostate cancer cells may be due to increased $p75^{NTR}$ mRNA instability. Consistently, I found that the observed increase in $p75^{NTR}$ protein due to R-flurbiprofen and ibuprofen treatment was accompanied by an increase in $p75^{NTR}$ mRNA, and this increase in mRNA was the result of increased $p75^{NTR}$ mRNA stability. In addition, treatment with R-flurbiprofen or ibuprofen led to sustained activation of the p38 MAPK pathway and inhibition of this pathway prevented an induction of $p75^{NTR}$ by R-flurbiprofen and ibuprofen. Collectively, the data suggest that R-flurbiprofen and ibuprofen induce $p75^{NTR}$ expression leading to $p75^{NTR}$ dependent decreased survival by increased $p75^{NTR}$ mRNA stability that is mediated through the p38 MAPK pathway. All procedures and results are described in detail in the manuscripts found in the appendices.

Task 2: The goal of Task 2 is to investigate the role of the aryl hydrocarbon receptor (AhR) in the induction of $p75^{NTR}$ expression by R-flurbiprofen or ibuprofen. With completion of the first aim of this project, I satisfied the requirements of the doctoral program for Georgetown University's Department of Biochemistry and Molecular & Cellular Biology. Therefore, although the second aim of this project is unfinished, I plan to graduate in December of 2007.

Key Research Accomplishments

All of the items listed in the Statement of Work Task 1, shown below, have been completed. Task 2 has not yet been completed. These and other research accomplishments have been published in the following two journal articles and are included as Appendices.

Quann EJ, Khwaja F, Zavitz KH, Djakiew D. The Aryl Propionic Acid R-Flurbiprofen Selectively Induces $p75^{NTR}$-Dependent Decreased Survival of Prostate Tumor Cells. Cancer Res 2007;67:3254-62.

Quann EJ, Khwaja F, Djakiew D. The p38 MAPK Pathway Mediates Aryl Propionic Acid-Induced Messenger RNA Stability of $p75^{NTR}$ in Prostate Cancer Cells. Cancer Res 2007;67:11402-10.

Task 1: Determine the role of $p75^{NTR}$ induction in aryl propionic acid induced survival inhibition of prostate cancer cells (1-18 months).

- Perform western blots for cyclooxygenase (COX) expression following 48 hour aryl propionic acid treatment of PC-3 or DU-145 cells.

- Perform western blots for expression of other tumor necrosis factor receptor (TNFR) superfamily members following 48 hour aryl propionic acid treatment of PC-3 or DU-145 cells.

- Perform western blots for $p75^{NTR}$ following 2 week chronic aryl propionic acid treatment of PC-3 or DU-145 cells at concentrations lower than those used in the 48 hour treatments.

- Determine enumeration of PC-3 and DU-145 cells using a hemocytometer following 2 week chronic aryl propionic acid treatment at concentrations lower than those used in the 48 hour treatments.

- Perform rescue experiments with PC-3 and DU-145 cells involving transfection of ecdysone-inducible dominant negative $p75^{NTR}$ expression vectors followed by 48 hour aryl propionic acid treatment. Determine relative survival by MTT assay.

- Perform rescue experiments with PC-3 and DU-145 cells involving 24 hour transfection of $p75^{NTR}$-targeted siRNA followed by 48 hour aryl propionic acid treatment. Determine relative cell survival by MTT assay.

Reportable Outcomes

Manuscripts

- Quann EJ, Khwaja F, Zavitz KH, Djakiew D. The Aryl Propionic Acid R-Flurbiprofen Selectively Induces $p75^{NTR}$-Dependent Decreased Survival of Prostate Tumor Cells. Cancer Res 2007;67:3254-62.

- Quann EJ, Khwaja F, Djakiew D. The p38 MAPK Pathway Mediates Aryl Propionic-Acid Induced Messenger RNA Stability of $p75^{NTR}$ in Prostate Cancer Cells. Cancer Res 2007;67:11402-10.

Conference Abstracts

- Quann EJ, Khwaja F, Djakiew D. The p38 MAPK pathway mediates aryl propionic acid induced mRNA stability of $p75^{NTR}$ in prostate cancer cells. AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics, October 2007, San Francisco.

Degrees Obtained

- Ph.D. Georgetown University, Biochemistry and Molecular & Cellular Biology

Employment Opportunities

- Postdoctoral position, Memorial Sloan-Kettering Cancer Center, Immunology Research Program

Conclusions

This study revealed that several aryl propionic acids induce reexpression of $p75^{NTR}$ in two different metastatic hormone-refractory prostate cancer cell lines, PC-3 and DU-145. Of those tested, the enantiomer R-flurbiprofen and ibuprofen were the most effective. These drugs were also effective in inducing $p75^{NTR}$ expression in the metastatic androgen responsive LNCaP cell line. R-flurbiprofen or ibuprofen treatment of all three cell lines resulted in decreased survival that corresponded with induction of $p75^{NTR}$, confirming the potential for these drugs as anticancer agents in the prostate and also suggesting that $p75^{NTR}$ induction is involved in the observed decreased survival. In addition, treatment with these drugs resulted in induction of apoptosis. Consistently, $p75^{NTR}$ is a TNFR family member capable of inducing apoptosis through its conserved intracellular death domain. There are several other TNFR family members that also possess apoptosis inducing death domains; however, none of these family members was significantly upregulated in response to profen treatment. Since the profens are traditionally used as COX inhibitors, the status of COX expression was determined in PC-3 and DU-145 cells. Both cell lines expressed very low levels of the housekeeping isoform COX-1, and expression remained constant with profen treatment. COX-2 expression was not detected in untreated or profen treated DU-145 cells. COX-2 expression was also not detected in untreated PC-3 cells, but was induced following profen treatment. This data and the fact that R-flurbiprofen is the enantiomer that lacks COX inhibitory activity indicate that the profens decrease survival and induce $p75^{NTR}$ independently of COX inhibition in prostate cancer cells. In order to determine if $p75^{NTR}$ induction is causal of the observed profen mediated decreased survival and increased apoptosis in prostate cancer cells, two dominant negative forms of $p75^{NTR}$ as well as $p75^{NTR}$ siRNA were employed. Transfection of PC-3 and DU-145 cells with either dominant negative form of $p75^{NTR}$ or $p75^{NTR}$ siRNA before R-flurbiprofen or ibuprofen treatment resulted in at least a partial rescue from profen mediated decreased survival in all cases. These results indicate that induction of $p75^{NTR}$ is at least partially responsible for mediating the anticancer effects of R-flurbiprofen and ibuprofen in these prostate cancer cells. Increases in $p75^{NTR}$ protein expression were found to closely correlate with increases in $p75^{NTR}$ mRNA following profen treatment. The observed increase in $p75^{NTR}$ mRNA level was found to be largely due to substantial increases in mRNA stability. The p38 MAPK pathway has been shown to regulate mRNA stability through its downstream kinases MK2 and MK3, and therefore, its role in $p75^{NTR}$ induction by profens was investigated. Profen treatment resulted in increased p38 MAPK phosphorylation, and inhibition of this pathway using a p38 MAPK inhibitor or by siRNA knockdown of p38 MAPK, or MK2 and MK3 before profen treatment prevented an induction of $p75^{NTR}$. Collectively, the data suggests that profen treatment activates the p38 MAPK pathway, which increases the stability of the $p75^{NTR}$ transcript, thus resulting in increased $p75^{NTR}$ mRNA, increased $p75^{NTR}$ protein expression, and $p75^{NTR}$ mediated decreased prostate cancer cell survival.

The results described above have several implications. They contribute to the body of evidence demonstrating the potential of aryl propionic acids for the treatment of prostate cancer, and identify induction of $p75^{NTR}$ as a COX independent mechanism by which these drugs achieve their anticancer activity in the prostate. This study also indicates that drugs which result in the reexpression of $p75^{NTR}$ may be effective in the treatment of advanced prostate cancer. It is significant that the profens were effective in inducing $p75^{NTR}$ expression and in decreasing survival of three different advanced prostate cancer cell lines, each derived from metastasis to a

different organ, as this demonstrates that the results are not an artifact of a single cell line. This is an important observation given the significant dedifferentiation and heterogeneity associated with the advanced disease. In addition, these results were observed in both androgen independent and androgen responsive cells lines, suggesting these drugs may be effective for advanced prostate cancer regardless of AR status. Finally, this brings to light novel approaches for identifying drugs which induce $p75^{NTR}$ with greater specificity. For example, identification of drugs that activate p38 MAPK may reveal drugs that induce $p75^{NTR}$. Better yet would be the identification of drugs which specifically activate the downstream kinases MK2 and MK3, as these may have decreased side effects relative to drugs that activate p38 MAPK which has many downstream targets. The exact mechanisms by which MK2 and MK3 stabilize mRNAs need to be explored further. It is highly likely that several RNA binding proteins are involved in increasing $p75^{NTR}$ mRNA stability due to profen treatment, and further elucidation of this mechanism may lead to identification of additional novel targets and the identification and development of therapeutics highly specific in inducing $p75^{NTR}$ in prostate cancer cells with minimal off target effects.

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Appendices

Quann EJ, Khwaja F, Zavitz KH, Djakiew D. The Aryl Propionic Acid R-Flurbiprofen Selectively Induces $p75^{NTR}$-Dependent Decreased Survival of Prostate Tumor Cells. Cancer Res 2007;67:3254-62.

Quann EJ, Khwaja F, Djakiew D. The p38 MAPK Pathway Mediates Aryl Propionic Acid-Induced Messenger RNA Stability of $p75^{NTR}$ in Prostate Cancer Cells. Cancer Res 2007;67:11402-10.

The Aryl Propionic Acid *R*-Flurbiprofen Selectively Induces $p75^{NTR}$-Dependent Decreased Survival of Prostate Tumor Cells

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Abstract

Epidemiologic studies show that patients chronically consuming nonsteroidal anti-inflammatory drugs (NSAID) for arthritis exhibit a reduced incidence of prostate cancer. In addition, some NSAIDs show anticancer activity *in vitro*. NSAIDs exert their anti-inflammatory effects by inhibiting cyclooxygenase (COX) activity; however, evidence suggests that COX-independent mechanisms mediate decreased prostate cancer cell survival. Hence, we examined the effect of selected aryl propionic acid NSAIDs and structurally related compounds on the decreased survival of prostate cancer cell lines PC-3, DU-145, and LNCaP by induction of the $p75^{NTR}$ protein. $p75^{NTR}$ has been shown to function as a tumor suppressor in the prostate by virtue of its intracellular death domain that can initiate apoptosis and inhibit growth. The most efficacious compounds for induction of $p75^{NTR}$ and decreased survival, in rank-order, were *R*-flurbiprofen, ibuprofen, oxaprozin, fenoprofen, naproxen, and ketoprofen. Because *R*-flurbiprofen and ibuprofen exhibited the greatest efficacy, we examined their dose-dependent specificity of induction for $p75^{NTR}$ relative to other members of the death receptor family. Whereas treatment with *R*-flurbiprofen or ibuprofen resulted in a massive induction of $p75^{NTR}$ protein levels, the expression of Fas, $p55^{TNFR}$, DR3, DR4, DR5, and DR6 remained largely unchanged. Moreover, transfection of either cell line before *R*-flurbiprofen or ibuprofen treatment with a dominant negative form of $p75^{NTR}$ to antagonize $p75^{NTR}$ activity or $p75^{NTR}$ small interfering RNA to prevent $p75^{NTR}$ protein expression rescued both cell lines from decreased survival. Hence, *R*-flurbiprofen and ibuprofen selectively induce $p75^{NTR}$-dependent decreased survival of prostate cancer cells independently of COX inhibition. [Cancer Res 2007;67(7):3254–62]

Introduction

Prostate cancer is the most commonly diagnosed cancer and ranks as the second leading cause of cancer-related deaths among men in the United States (1, 2). Recent epidemiologic studies found a correlation between long-term nonsteroidal anti-inflammatory drug (NSAID) use and decreased prostate cancer risk (3–6). In addition, many *in vitro* studies involving various human prostate cancer cell lines consistently show decreased proliferation and increased apoptosis in response to select NSAID treatment (7, 8). NSAIDs exert their anti-inflammatory activity by inhibiting cyclooxygenase (COX), the enzyme which catalyzes the conversion of arachidonic acid to prostaglandins. Two isoforms of COX exist; COX-1 is a housekeeping gene that is constitutively expressed at low levels in most cells types, whereas COX-2 is highly inducible in response to cytokines, hormones, and growth factors. COX-2 seems to play a significant role in the promotion of colon cancer with 50% of precancerous adenomatous polyps and 85% of colon carcinomas exhibiting COX-2 overexpression (9). However, the data pertaining to the role of COX-2 in prostate cancer are less conclusive. Although some studies show overexpression, others show expression is low or absent relative to normal tissue (10–14). In addition, there is not a consensus regarding the status of COX-2 expression in established prostate cancer cell lines, including LNCaP, DU-145, and PC-3 (13, 15–17). However, regardless of COX-2 expression, these cell lines all show susceptibility to select NSAID treatment (17–19). Furthermore, the anticancer activity of NSAIDs occurs at concentrations several orders of magnitude higher than those required to inhibit COX-2, and different NSAIDs show varying levels of anticancer activity (20–22). These results suggest the existence of a COX-independent mechanism by which some NSAIDs initiate apoptosis and inhibit proliferation.

The $p75^{NTR}$ (neurotrophin receptor) is a member of the tumor necrosis factor receptor (TNFR) superfamily capable of inducing apoptosis through a conserved intracellular death domain (23, 24). It binds neurotrophin ligands with similar affinity; however, unlike other members of the TNFR superfamily, $p75^{NTR}$ induces cell death and suppresses growth in the unbound state (25–29). Recently, $p75^{NTR}$ was identified as a tumor and metastasis suppressor in the prostate and bladder (27, 28). Although normal prostate epithelial cells express high levels of $p75^{NTR}$, this expression becomes suppressed as prostate cancer progresses (30). In addition, the human prostate cancer cell lines PC-3, DU-145, and LNCaP, all derived from metastases, show little to no $p75^{NTR}$ expression (31). However, the $p75^{NTR}$ gene in these cells has remained intact, indicating that the potential for up-regulation may exist (31). Furthermore, exogenous reexpression of $p75^{NTR}$ in PC-3 cells suppressed growth and increased apoptosis (28, 32). This suggests potential for treatments that result in the up-regulation and reexpression of $p75^{NTR}$ in prostate cancer cells.

Ibuprofen and flurbiprofen belong to the aryl propionic acid class of NSAIDs. Treatment of T24 bladder cancer cells and HCT-116 colon cancer cells with ibuprofen or the enantiomer *R*-flurbiprofen, which lacks COX inhibitory activity, induced a dose-dependent up-regulation of $p75^{NTR}$ and a corresponding decrease in survival (33). Rescue experiments involving transfection of dominant negative forms of $p75^{NTR}$ before ibuprofen treatment of T24 cells showed that the observed induction of $p75^{NTR}$ was causal of the decreased survival (33). Significantly, *R*-flurbiprofen treatment of TRAMP mice has been shown to lower the incidence of primary tumors and metastases of prostate cancer (34). In this context, we show that *R*-flurbiprofen and ibuprofen most

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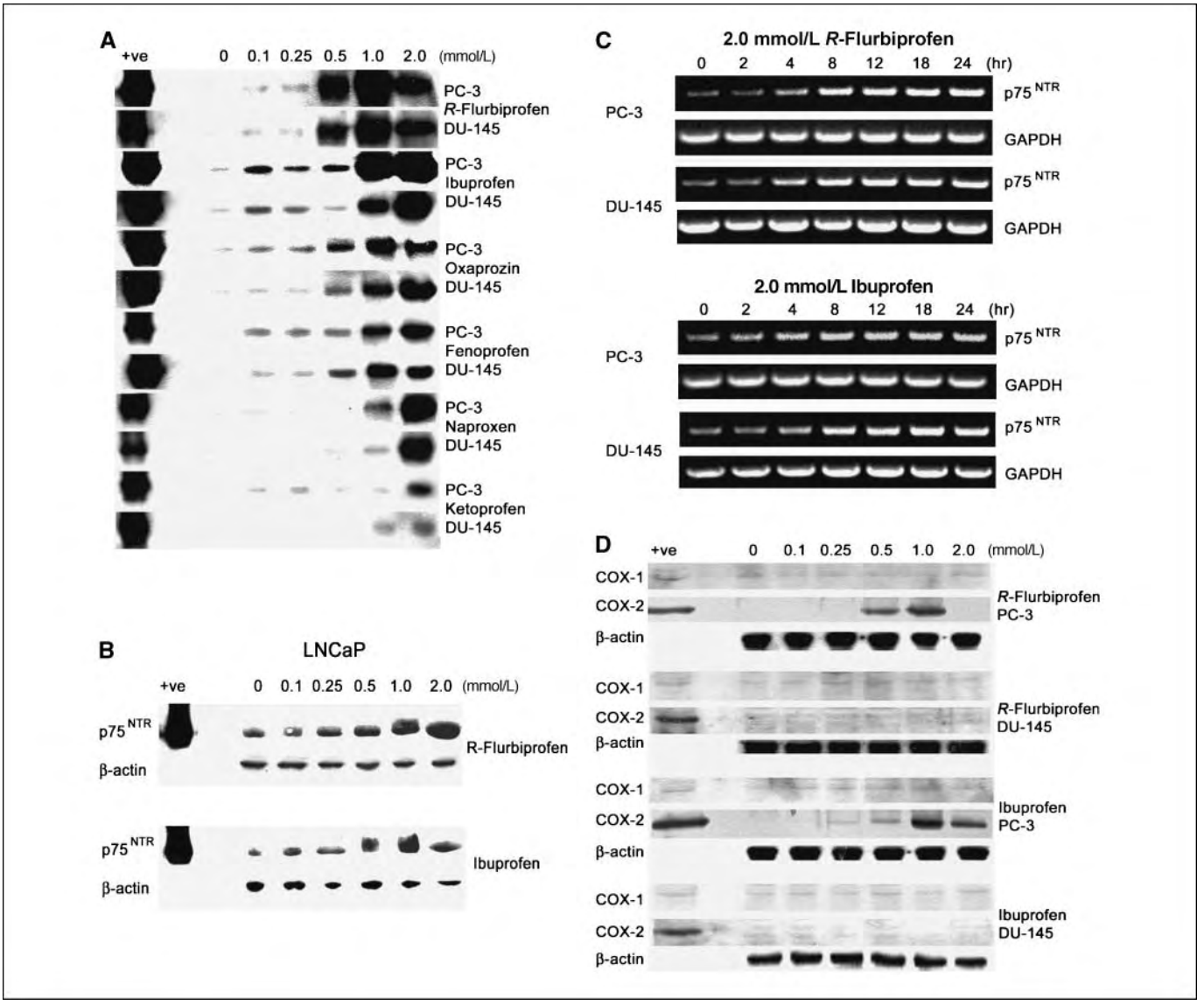

Figure 1. *A,* expression of the $p75^{NTR}$ in PC-3 and DU-145 cells following 48-h treatment with 0, 0.1, 0.25, 0.5, 1.0, or 2.0 mmol/L *R*-flurbiprofen, ibuprofen, oxaprozin, fenoprofen, naproxen, or ketoprofen. Cell lysates were collected, and 50 μg of protein were subjected to SDS-PAGE and immunoblot analysis using a monoclonal antibody to human $p75^{NTR}$. The A875 melanoma cell line was used as a positive control (*+ve*). *B,* expression of $p75^{NTR}$ in LNCaP cells following 48-h treatment with 0 to 2 mmol/L *R*-flurbiprofen or ibuprofen. SDS-PAGE was done using 50 μg of protein followed by immunoblot analysis. *C,* $p75^{NTR}$ and GAPDH mRNA levels in PC-3 and DU-145 cells following 0, 2, 4, 8, 12, 18, and 24 h of 2.0 mmol/L *R*-flurbiprofen or ibuprofen treatment determined by RT-PCR. *D,* expression of COX-1 and COX-2 in PC-3 and DU-145 cells following 48-h treatment with 0, 0.1, 0.25, 0.5, 1.0, or 2.0 mmol/L *R*-flurbiprofen or ibuprofen. SDS-PAGE was done using cell lysates containing 50 μg of protein and followed by immunoblot analysis using a monoclonal antibody to human COX-1 and goat polyclonal antibody to human COX-2. U-937 and RAW 264.7 cell lysates were used as positive controls for COX-1 and COX-2, respectively.

effectively up-regulate $p75^{NTR}$ expression in both PC-3 and DU-145 cell lines relative to other aryl propionic acids. This up-regulation occurs in a dose-dependent manner and corresponds to a decrease in cell survival. The use of $p75^{NTR}$ dominant negatives and $p75^{NTR}$-targeting small interfering RNA (siRNA) shows that the observed decreased survival is directly mediated through $p75^{NTR}$, a COX-independent mechanism.

Materials and Methods

Cell lines and culture conditions. PC-3 and DU-145 cell lines were obtained from the tissue culture core facility of the Georgetown University Lombardi Comprehensive Cancer Center. LNCaP cells were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were maintained in DMEM (Mediatech Inc., Herndon, VA) containing 4.5 g/L glucose and L-glutamine supplemented with antibiotic/antimycotic [100 units/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotercin B (Mediatech Inc.)] and 5% fetal bovine calf serum (Sigma Chemical Co., St. Louis, MO). Cells were incubated in the presence of 5% CO_2 and air at 37°C.

Drug preparation, treatment, and cell lysis. Stock solutions were prepared by dissolving each aryl propionic acid [ibuprofen, ketoprofen, naproxen, oxaprozin, fenoprofen (Sigma), and *R*-flurbiprofen (Myriad Pharmaceuticals Inc., Salt Lake City, UT)] in DMSO (Sigma) at a concentration of 200 mmol/L. Cells were seeded overnight at 70% to 80% confluency and were then treated for 48 h at concentrations of 0, 0.1, 0.25, 0.5, 1.0, and 2.0 mmol/L. Cell lysates of treated cells were prepared using lysis buffer [10 mmol/L Tris-Cl (pH, 7.4), 10 mmol/L NaCl, 3 mmol/L $MgCl_2$, and 0.5% Nonidet P-40] containing 1 μL/mL cocktail protease inhibitor

(Sigma). The supernatant was retained, and protein concentration was determined according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA). For long-term treatment of cells, ~1000 cells were seeded overnight and treated the next day with 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mmol/L ibuprofen or *R*-flurbiprofen twice per week for 2 weeks. After 2 weeks, lysates were prepared as described above, or cells were trypsinized, resuspended, and counted using a hemocytometer.

Immunoblot analysis. Immunoblot analysis was done by loading 50 μg of protein onto 10% SDS-polyacrylamide gels for electrophoresis, followed by transfer to a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were blocked in 5% nonfat milk (Bio-Rad Laboratories) and then incubated in the primary antibody: murine monoclonal anti-$p75^{NTR}$ (1:2,000, Upstate Cell Signaling Solutions, Lake Placid, NY), murine monoclonal anti-Fas (1:100, Santa Cruz Biotechnologies, Santa Cruz, CA), murine monoclonal anti-$p55^{TNFR}$ (1:100, Santa Cruz Biotechnologies), rat polyclonal anti-DR3 (1:200, Santa Cruz Biotechnologies), murine monoclonal anti-DR4 (1:100, Santa Cruz Biotechnologies), rat polyclonal anti-DR5 (1:500, ProSci Inc., Poway, CA), rat polyclonal anti-DR6 (2 μg/mL; Upstate Cell Signaling Solutions), mouse monoclonal anti–COX-1 (1:200, Santa Cruz Biotechnologies), goat polyclonal anti–COX-2 (1:200, Santa Cruz Biotechnologies), or murine monoclonal anti–β-actin (1:5000, Sigma). Following incubation in the primary antibody, membranes were incubated in the appropriate horseradish peroxidase–conjugated secondary antibody (1:3,000, Bio-Rad Laboratories). Immunoreactivity was detected using the chemiluminescence detection reagent (Amersham Pharmacia Biotech). Several positive controls were used: A875 whole cell lysate (WCL; Tissue Culture Core Facility, Georgetown University) for $p75^{NTR}$, A431 WCL (Santa Cruz Biotechnologies) for Fas, MCF-7 WCL (Santa Cruz Biotechnologies) for $p55^{TNFR}$, Jurkat WCL (Upstate Cell Signaling Solutions) for DR3 and DR6, HeLa WCL (ProSci Inc.) for DR4 and DR5, U-937 WCL (Santa Cruz Biotechnologies) for COX-1, and RAW 264.7 WCL (Santa Cruz Biotechnologies) for COX-2.

Reverse transcription-PCR. PC-3 and DU145 cells were treated with 2.0 mmol/L *R*-flurbiprofen or ibuprofen. RNA was isolated following 0, 2, 4, 8, 12, 18, and 24 h of treatment using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Reverse transcription-PCR (RT-PCR) was done using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen) using 250 ng RNA for $p75^{NTR}$ and 125 ng RNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers were designed using Primer Quest, and their sequences are as follows: $p75^{NTR}$ forward 5′-AGG TGA CCT TCT GGG AAA TGG CTT-3′, $p75^{NTR}$ reverse 5′-ATT TCC TCC GAT GCT TCT CTG GCA-3′, GAPDH forward 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′, and GAPDH reverse 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′ (Integrated DNA Technologies, Coralville, IA). cDNA synthesis was done at 47°C for 30 min followed by denaturation at 94°C for 2 min and then 30 cycles of PCR at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with final extension at 72°C for 5 min. PCR products were separated on 1.5% agarose gels.

Hoechst dye nuclear (DNA) staining. Hoechst staining to identify apoptotic nuclei was conducted as described previously (33). PC-3, DU-145, and LNCaP cells were treated for 48 h with aryl propionic acids and then fixed in 10% formalin (Electron Microscopy Sciences, Hatfield, PA). Cells were collected, washed in PBS, centrifuged, resuspended in PBS, and dried on slides. Slides were rehydrated with PBS, washed with distilled water, and covered with a 1:1,000 aqueous dilution of Hoechst 33258 stain (Molecular Probes, Eugene, OR) for a final concentration of 10 μg/mL. After 10 min, slides were washed with distilled water, mounted, and viewed using a fluorescence microscope (Zeiss Axioplan 2 Imaging, Jena, Germany).

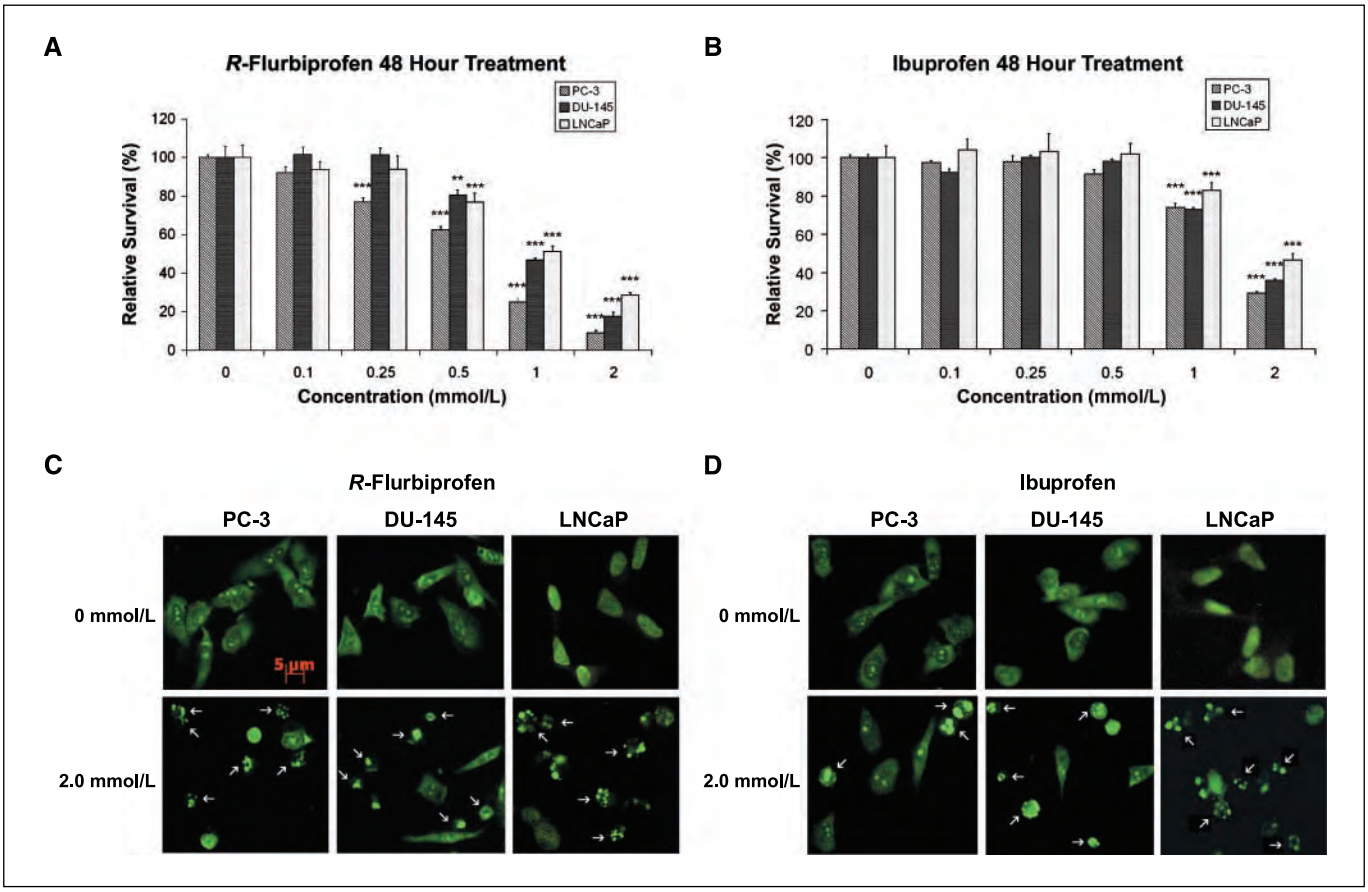

Figure 2. *A* and *B,* PC-3, DU-145, and LNCaP cell survival analysis by MTT assay following treatment with 0, 0.1, 0.25, 0.5, 1.0, or 2.0 mmol/L *R*-flurbiprofen or ibuprofen for 48 h. *Columns,* mean relative to the control (0 mM); *bars,* SE. **, $P < 0.01$; ***, $P < 0.001$. *C* and *D,* detection of apoptotic nuclei (*arrows*) by Hoechst staining of PC-3, DU-145, and LNCaP cells following treatment with 0 or 2.0 mmol/L *R*-flurbiprofen or ibuprofen.

p75^{NTR} dominant negative transfection. PC-3 and DU-145 cells were transiently transfected with one of two p75^{NTR} dominant negative vectors described previously (35). The ΔDD vector expresses p75^{NTR} with the death domain deleted, and the ΔICD vector expresses p75^{NTR} with the slightly larger intracellular domain deleted. Both are ecdysone-inducible p75^{NTR} vectors and, therefore, were each cotransfected with the ecdysone receptor plasmid pVgRxR. The transfection was done with LipofectAMINE reagent (Invitrogen) in serum-free medium for 6 h, after which serum-containing medium was added. After 18 subsequent hours, cells were incubated in 1 μmol/L ponasterone A (Invitrogen) for 24 h to drive the expression of the dominant negative gene products. Following incubation with ponasterone A, cells were treated with aryl propionic acids for 48 h, and relative cell survival was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Applied Science, Indianapolis, IN).

siRNA transfection. PC-3 and DU-145 cells were transfected for 24 h with nontargeting or p75^{NTR}-specific siRNA [Dharmacon RNA Technologies (Duplex D-009340-03), Lafayette, CO] at a final concentration of 100 nmol/L according to the manufacturer's protocol using transfection reagents DharmaFECT 1 for DU-145 cells and DharmaFECT 2 (Dharmacon RNA Technologies) for PC-3 cells. After transfection, the cells were treated with aryl propionic acids for 48 h, followed by determination of p75^{NTR} protein expression or relative cell survival by MTT assay (Roche Applied Science).

Results

Aryl propionic acids selectively induce p75^{NTR} expression and decrease cell survival. We examined the ability of *R*-flurbiprofen and ibuprofen, as well as four other members of the aryl propionic acid family, including oxaprozin, fenoprofen, naproxen, and ketoprofen to induce the expression of p75^{NTR} protein in PC-3 and DU-145 human prostate cancer cells. In both cell lines, *R*-flurbiprofen was the most efficacious for the induction of p75^{NTR} expression followed by ibuprofen, oxaprozin, fenoprofen, naproxen, and finally, ketoprofen, which was the least effective compound (Fig. 1*A*). For subsequent experiments, only *R*-flurbiprofen and ibuprofen were used because they were the most effective of the six aryl propionic acids tested. Treatment with *R*-flurbiprofen or ibuprofen also resulted in an induction of p75^{NTR} in LNCaP cells, which are androgen responsive (Fig. 1*B*). To elucidate the mechanism by which these aryl propionic acids may be inducing p75^{NTR} protein expression, we determined relative p75^{NTR} mRNA levels at various time points following 2.0 mmol/L *R*-flurbiprofen or ibuprofen treatment (Fig. 1*C*). In both cell lines, *R*-flurbiprofen and ibuprofen significantly induced p75^{NTR} message level. This induction is first noticeable within 4 h of treatment in both cell lines with both drugs.

The aryl propionic acids are commonly used as NSAIDs that act through COX inhibition. Therefore, we examined the status of COX expression in both cell lines and determined whether the expression level of either isoform changed in response to treatment with *R*-flurbiprofen and ibuprofen (Fig. 1*D*). Both PC-3 and DU-145 cells expressed COX-1 at low levels, and expression remained unchanged following *R*-flurbiprofen or ibuprofen treatment. PC-3 cells lacked COX-2 expression; however, expression was induced upon treatment with *R*-flurbiprofen or ibuprofen. DU-145 cells also lacked COX-2 expression, and no induction was observed following treatment with either compound.

Examination of the effect of *R*-flurbiprofen and ibuprofen on survival of PC-3, DU-145, and LNCaP cells after 48-h treatment resulted in a dose-dependent decrease in survival that corresponded with the observed induction of p75^{NTR} (Fig. 2*A* and *B*). Again, *R*-flurbiprofen was more efficacious than ibuprofen, resulting in a greater dose-dependent decrease in survival. Because p75^{NTR} contains an intracellular death domain capable of initiating apoptosis, we used Hoechst staining to identify fragmented nuclei typical of apoptotic cells. Treatment with 2.0 mmol/L *R*-flurbiprofen or ibuprofen resulted in an induction of apoptotic cells, indicating that apoptosis was at least partially responsible for the observed decrease in survival (Fig. 2*C* and *D*).

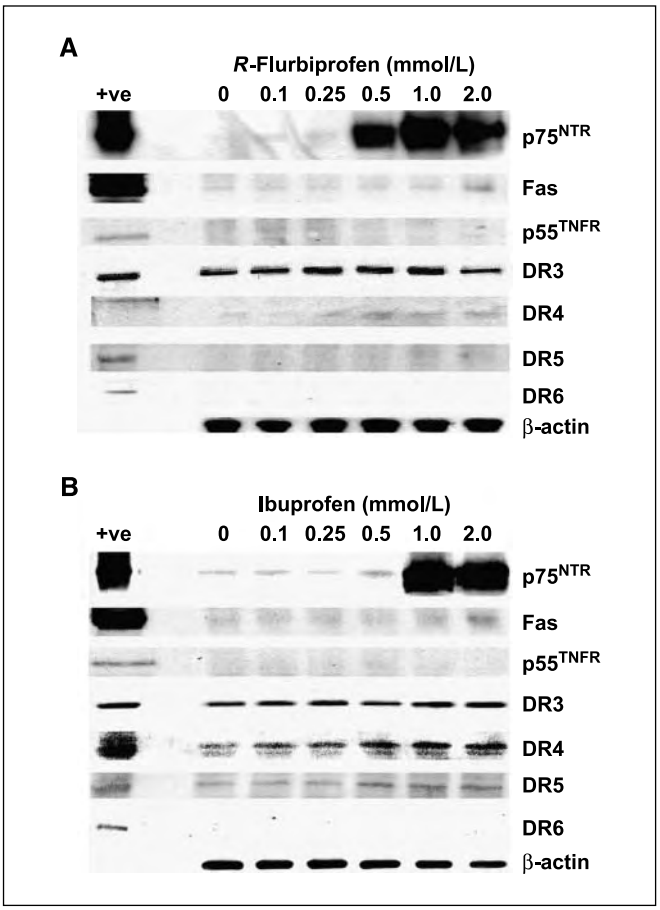

Figure 3. *A* and *B,* expression of TNFR superfamily members in PC-3 cells after 48-h treatment with 0, 0.1, 0.25, 0.5, 1.0, or 2.0 mmol/L *R*-flurbiprofen or ibuprofen. 50 μg of cell lysate were subjected to SDS-PAGE followed by immunoblot analysis using monoclonal antibodies to human Fas, p55^{TNFR}, and DR4, or rat polyclonal antibodies to human DR3, DR5, and DR6, and β-actin as the loading control. A431, MCF-7, Jurkat, and HeLa cell lysates were used as positive controls for Fas, p55^{TNFR}, DR3 and DR6, and DR4 and DR5, respectively.

p75^{NTR} is a member of the TNFR superfamily whose members contain an intracellular death domain capable of inducing apoptosis (23, 24). Although 48-h treatment with *R*-flurbiprofen resulted in a substantial dose-dependent induction of p75^{NTR}, the other death receptors, including Fas, p55^{TNFR}, DR3, DR4, DR5, and DR6, showed little, if any, induction in PC-3 cells (Fig. 3*A*). Similar results were observed following treatment with ibuprofen (Fig. 3*B*). There seemed to be a slight induction of DR4 with ibuprofen; however, the magnitude of the response was small relative to the induction observed for p75^{NTR} expression (Fig. 3*B*). The addition of the ligand corresponding to each death receptor had no effect on the induction of death receptors by *R*-flurbiprofen or ibuprofen (data not shown). Therefore, *R*-flurbiprofen and ibuprofen selectively induced p75^{NTR}, whereas protein expression of the other TNFR family members remained largely unchanged.

Long-term *R*-flurbiprofen and ibuprofen treatment results in p75^{NTR} induction and decreased survival at lower drug concentrations. Epidemiologic studies have shown a correlation between long-term continuous NSAID use and a decreased risk of prostate cancer (3, 4). Therefore, we examined whether chronic treatment with *R*-flurbiprofen or ibuprofen for 2 weeks at lower concentrations of 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mmol/L may be equally effective or more effective than the 48-h acute treatment at higher concentrations in PC-3 and DU-145 cells. Long-term treatment with *R*-flurbiprofen resulted in a dose-dependent decrease in cell growth with severe growth arrest even at 0.1 mmol/L and almost a complete loss of growth at 0.3 mmol/L for PC-3 cells and 0.5 mmol/L for DU-145 cells (Fig. 4*A*). In addition, there was a dose-dependent increase in p75^{NTR} levels beginning at 0.1 mmol/L, which corresponds to the concentration at which decreased growth first occurred (Fig. 4*B*). Similarly, long-term treatment with ibuprofen resulted in a dose-dependent decrease in survival and induction of p75^{NTR} at lower concentrations than observed with 48-h ibuprofen treatment, but was not as efficacious as long-term treatment with *R*-flurbiprofen (Fig. 4*C* and *D*).

Decreased survival caused by *R*-flurbiprofen and ibuprofen is mediated through p75^{NTR}. The results presented thus far show that the induction of p75^{NTR} is associated with decreased survival of PC-3 and DU-145 cells in response to *R*-flurbiprofen or ibuprofen treatment. To determine if the observed decreased survival is causally mediated through *R*-flurbiprofen– and ibuprofen-dependent induction of p75^{NTR}, ponasterone A–inducible expression vectors for one of two different dominant negative forms of p75^{NTR} were transfected into PC-3 and DU-145 cells before *R*-flurbiprofen or ibuprofen treatment. The dominant negative vectors both express truncated forms of p75^{NTR}. ΔDDp75^{NTR} has a deletion of the death domain, and ΔICDp75^{NTR} has a deletion of the larger intracellular domain that includes the death domain. PC-3 and DU-145 cells treated with 0, 0.5, or 1.0 mmol/L *R*-flurbiprofen alone or with ponasterone A resulted in a similar dose-dependent decrease in survival (Fig. 5*A* and *B*). *R*-flurbiprofen treatment of cells transfected with either ΔDDp75^{NTR} or ΔICDp75^{NTR} but not treated with ponasterone A yielded the same result (Fig. 5*A* and *B*). However, cells transfected with either of the two dominant negative expression vectors followed by ponasterone A treatment to induce expression of the gene product exhibited increased survival following *R*-flurbiprofen treatment compared with cells not expressing dominant negative p75^{NTR} (Fig. 5*A* and *B*). Similar results were observed with ibuprofen in PC-3 and DU-145 cells, where expression of the dominant negative forms of p75^{NTR} partially prevented decreased survival due to ibuprofen (Fig. 5*C* and *D*).

We did similar rescue experiments using siRNA targeted against p75^{NTR}. Transfection of PC-3 cells with p75^{NTR} siRNA before *R*-flurbiprofen treatment almost completely prevented the induction of p75^{NTR} protein, whereas transfection with nontargeting siRNA did not prevent the induction of p75^{NTR} protein (Fig. 6*A*). Treatment with *R*-flurbiprofen at 0, 0.5, and 1.0 mmol/L in untransfected PC-3 cells or in PC-3 cells transfected with nontargeting siRNA resulted in a similar dose-dependent decrease in cell viability (Fig. 6*A*). However, treatment of PC-3 cells transfected with p75^{NTR} siRNA partially prevented *R*-flurbiprofen–dependent decreased survival at 0.5 and 1.0 mmol/L concentrations (Fig. 6*A*). Similarly, transfection of PC-3 or DU-145 cells with p75^{NTR}-targeting siRNA before ibuprofen treatment also almost completely blocked the induction of p75^{NTR}, whereas transfection with nontargeting siRNA did not block induction (Fig. 6*C* and *D*). Treatment with 0, 0.5, and 1.0 mmol/L ibuprofen

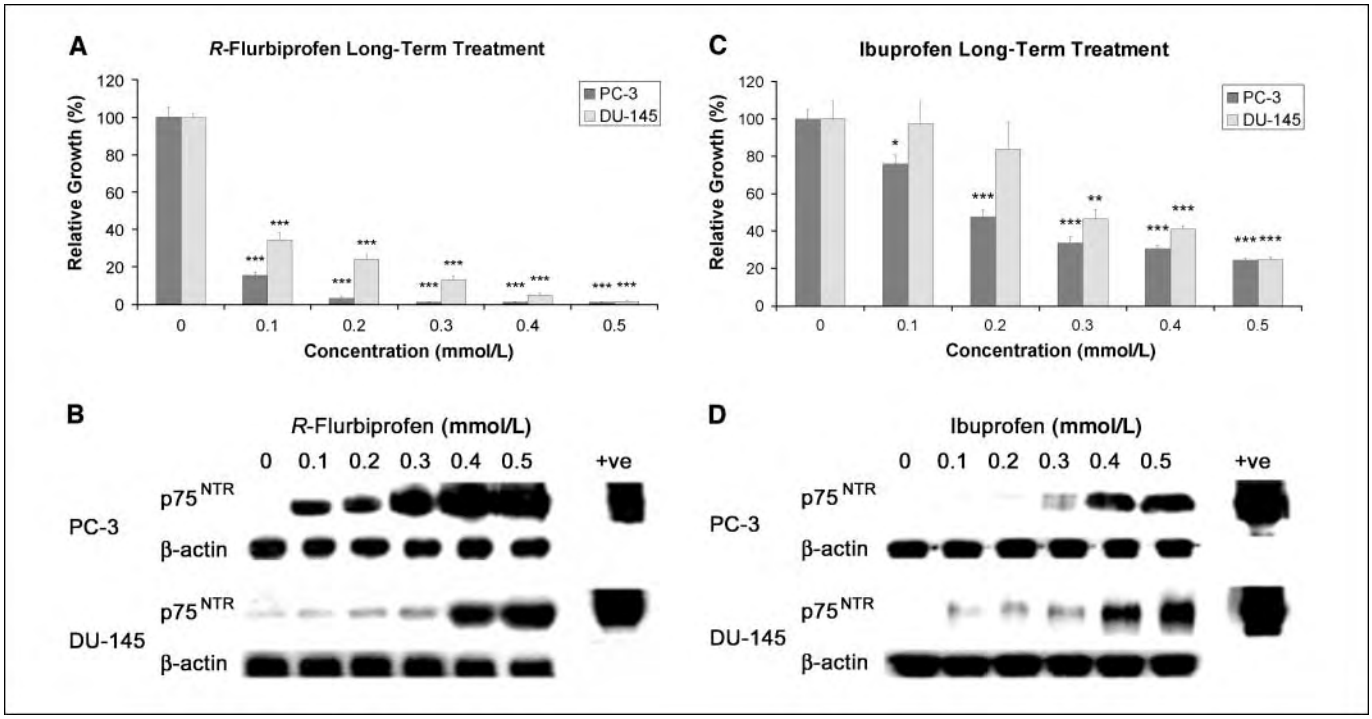

Figure 4. *A* and *C,* relative growth of PC-3 and DU-145 cells following treatment with 0, 0.1, 0.2, 0.3, 0.4, or 0.5 mmol/L *R*-flurbiprofen or ibuprofen twice per week for 2 wks. Cell number was determined using a hemocytometer. *Columns,* mean relative to the control (0 mM); *bars,* SE. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. *B* and *D,* expression of p75^{NTR} in PC-3 and DU-145 cells that were treated with 0, 0.1, 0.2, 0.3, 0.4, or 0.5 mmol/L *R*-flurbiprofen or ibuprofen twice per week for 2 wks. The A875 cell line was used as a positive control. SDS-PAGE was done using 50 μg of cell lysate and followed by immunoblot analysis using a monoclonal antibody to human p75^{NTR} and β-actin as the loading control.

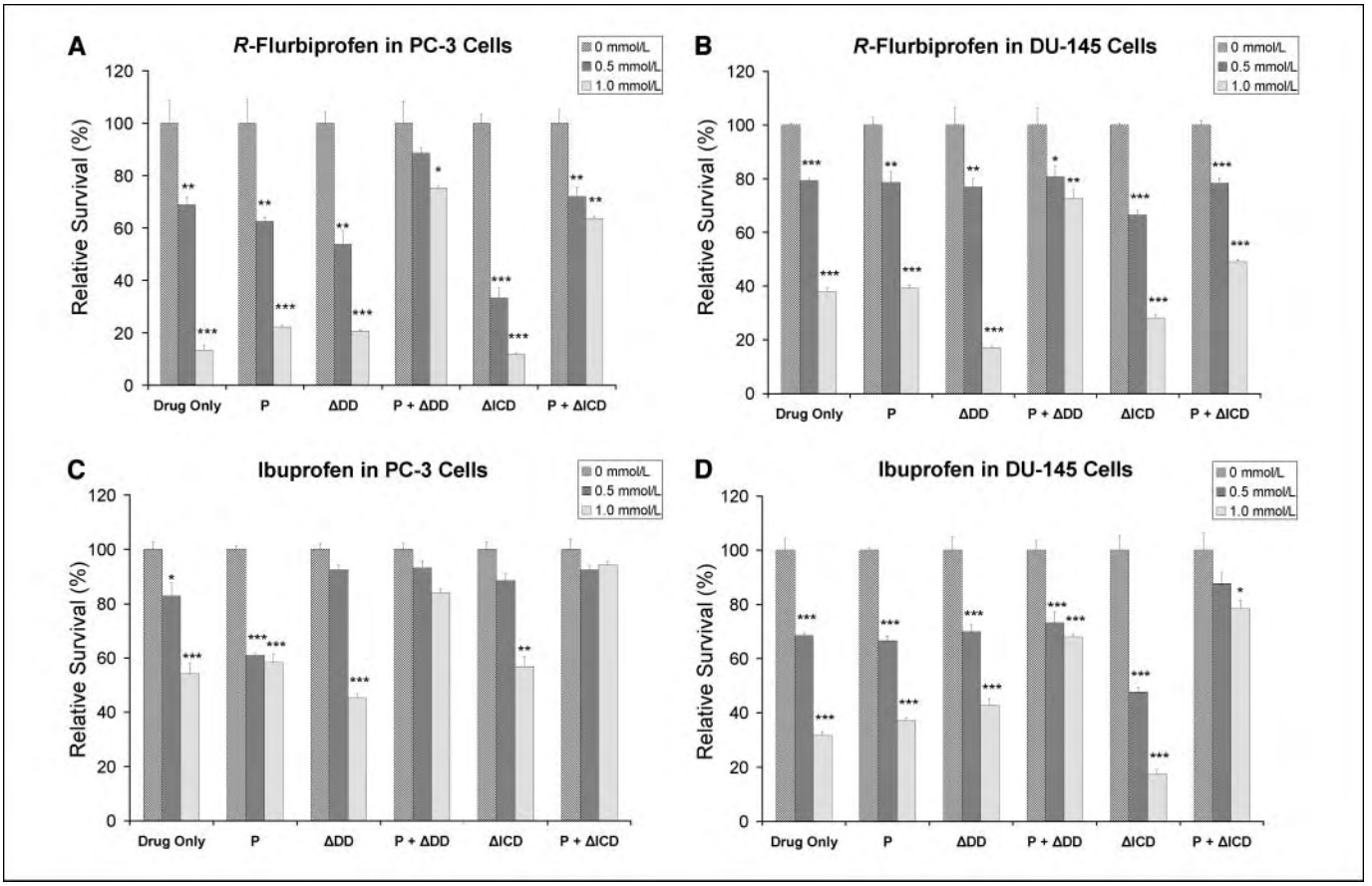

Figure 5. *A* to *D,* PC-3 and DU-145 cell survival analysis by MTT assay following 48-h treatment with 0, 0.5, or 1.0 mmol/L *R*-flurbiprofen or ibuprofen. Before treatment, cells were cotransfected for 6 h in serum-free medium with ponasterone A–inducible ecdysone receptor plasmid pVgRxR and either ΔDDp75^{NTR} or ΔICDp75^{NTR}, which express a truncated dominant negative p75^{NTR} protein that lacks either the death domain or the entire intracellular domain, respectively. Following transfection, cells were incubated in serum-containing medium for 18 h and then incubated in 1 μmol/L ponasterone A (P) for 24 h to drive expression of the dominant negative gene products. *Columns,* mean relative to the control (0 mM); *bars,* SE. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

led to a dose-dependent decrease of cell survival in untransfected cells and in cells transfected with nontargeting siRNA, whereas transfection with p75^{NTR} siRNA before ibuprofen treatment prevented a decrease in survival at the 1.0 mmol/L concentration (Fig. 6*C* and *D*). Transfection of DU-145 cells with p75^{NTR}-targeting siRNA only partially prevented p75^{NTR} induction upon *R*-flurbiprofen treatment (Fig. 6*B*). Consistently, only a partial rescue from *R*-flurbiprofen–dependent decreased survival was observed in these cells.

Discussion

The profens, also referred to as 2-aryl propionic acid derivatives, are a class of NSAIDs that share several characteristics. They can be reversible inhibitors of COX, are highly bound to plasma albumin, and are weak acids (36). They exist as enantiomer pairs, and it is generally the *S*-enantiomer, but not the *R*-enantiomer, that possesses potent COX inhibitory activity (37). Some profens have been in use for roughly 30 years, most commonly as treatment for inflammation due to rheumatoid arthritis (38–43). However, based on emerging studies associating NSAIDs with anticancer activity, various profens have been examined for their efficacy as chemopreventive and chemotherapeutic agents in a variety of cancer types (44–46). In this study, we examined the ability of six profens, including *R*-flurbiprofen, ibuprofen, oxaprozin, fenoprofen, naproxen, and ketoprofen, to induce the expression of the p75^{NTR} tumor suppressor in prostate cancer cells. We observed a dose-dependent induction of p75^{NTR} by these drugs; however, the level of efficacy varied with *R*-flurbiprofen followed by ibuprofen as the most effective. Consistently, previous reports identify these two drugs as promising agents in prostate cancer treatment. *R*-flurbiprofen was shown to inhibit the progression of prostate cancer in the TRAMP mouse, whereas ibuprofen was shown to reduce survival of LNCaP and DU-145 human prostate cancer cells (8, 21, 34). In addition, treatment of the human colon cancer COX-null cell line HCT-116 with either of these drugs resulted in reduced cell survival, indicating that these drugs possess the ability to inhibit growth through a COX-independent mechanism (33). The remaining four profens induced p75^{NTR} to a lesser degree with oxaprozin as the next most effective followed by fenoprofen, naproxen, and ketoprofen as the least effective. Of these four drugs, naproxen has also been shown to suppress growth of prostate cancer cells; however, it was less effective when compared with ibuprofen (21).

The results showed that an induction of p75^{NTR} was associated with significantly decreased survival of PC-3, DU-145, and LNCaP cells. Although these cell lines exhibited low levels of p75^{NTR} expression at lower concentrations of *R*-flurbiprofen and ibuprofen, the levels of p75^{NTR} were below the threshold for decreased survival. As the concentration of *R*-flurbiprofen or ibuprofen increased, some variability between levels of p75^{NTR} and decreased

survival may have resulted from discordance around inflection points. At higher concentrations of *R*-flurbiprofen and ibuprofen, levels of $p75^{NTR}$ were concordant with decreased survival. This cause and effect relationship between higher levels of $p75^{NTR}$ and decreased survival was subsequently shown in the rescue experiments with dominant negatives and siRNA to $p75^{NTR}$.

The exact mechanism by which *R*-flurbiprofen and ibuprofen induce $p75^{NTR}$ protein expression remains under investigation. However, an examination of $p75^{NTR}$ mRNA levels by RT-PCR at various time points showed that treatment of PC-3 and DU-145 cells with 2.0 mmol/L *R*-flurbiprofen or ibuprofen resulted in a relatively strong induction of $p75^{NTR}$ mRNA in all cases within 4 h and continuing until between 8 and 12 h, after which $p75^{NTR}$ mRNA remained constant. These results suggest that the observed induction of $p75^{NTR}$ protein expression is a result of an increase in $p75^{NTR}$ mRNA. This may occur by an increase in mRNA stability because previous work showed that $p75^{NTR}$ protein expression is lost in prostate cancer cell lines due to increased mRNA instability that is mediated through the 3′ untranslated region (31). Therefore, it is possible that treatment with *R*-flurbiprofen or ibuprofen alleviates this instability.

COX-2 catalyzes the conversion of arachidonic acid to prostaglandins, which are associated with increased survival, decreased apoptosis, and promotion of angiogenesis (13). Hence, inhibition of COX-2 has been suggested as a mechanism by which NSAIDs decrease cancer cell survival. However, an increasing body of literature suggests that many NSAIDs act through a COX-independent mechanism to achieve anticancer activity in the prostate (13). To elucidate whether COX plays a role in aryl propionic acid–dependent decreased prostate cancer cell survival, we examined the status of COX-1 and COX-2 expression. As expected, the housekeeping isoform COX-1 was expressed at low levels in PC-3 and DU-145 cells, and neither *R*-flurbiprofen nor ibuprofen induced its expression. Because COX-1 is not overexpressed in prostate cancer, is not generally associated with tumor development, and was minimally expressed in both cell lines at a

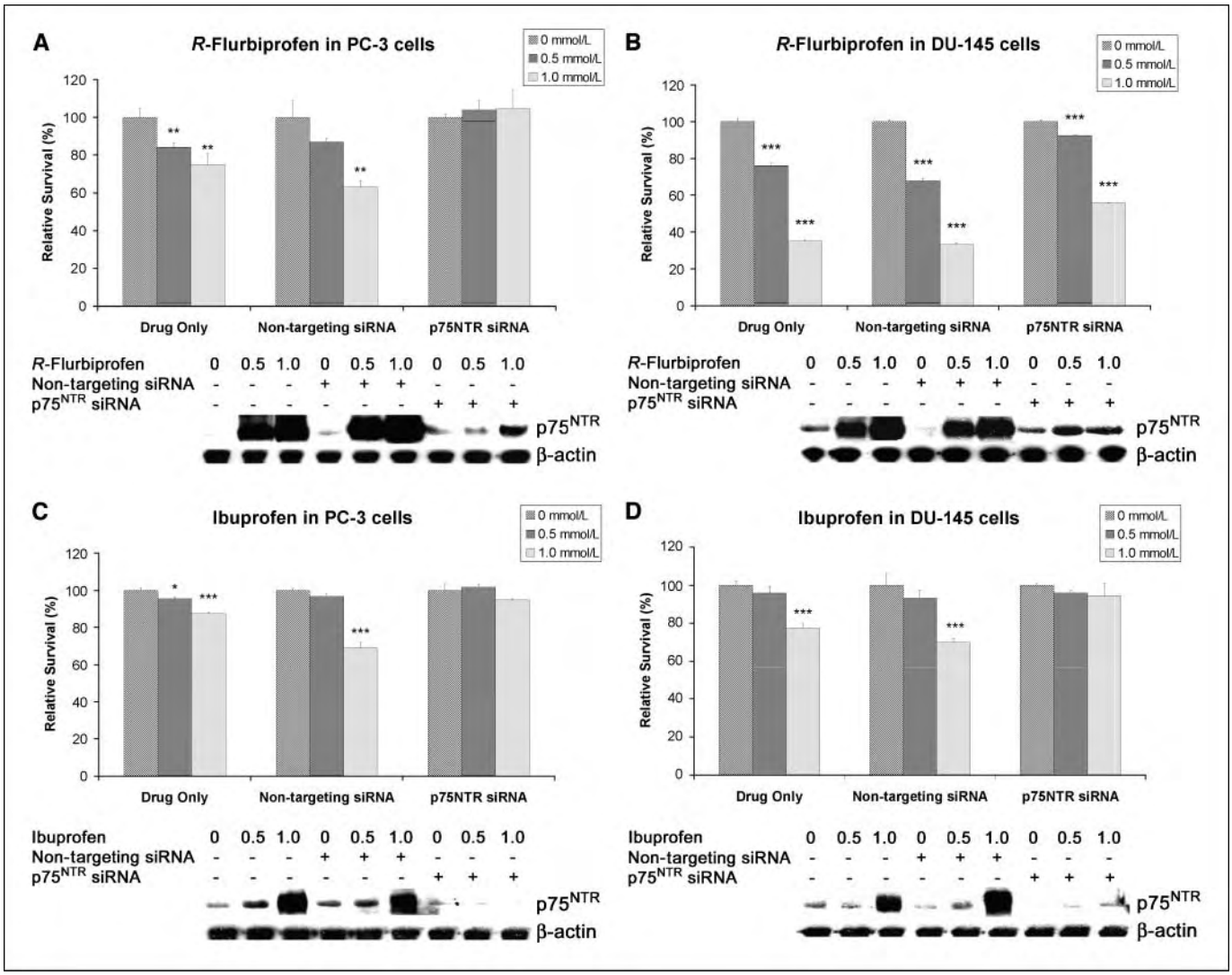

Figure 6. *A* to *D,* PC-3 and DU-145 cell survival analysis by MTT assay following 48-h treatment with 0, 0.5, or 1.0 mmol/L *R*-flurbiprofen or ibuprofen. Before treatment, cells were transfected with 100 nmol/L nontargeting siRNA or $p75^{NTR}$-targeting siRNA for 24 h. *Columns,* mean relative to the control (0 mM); *bars,* SE. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. $p75^{NTR}$ expression following 24-h transfection with 100 nmol/L nontargeting or $p75^{NTR}$-targeting siRNA and subsequent 48-h treatment with 0, 0.5, or 1.0 mmol/L *R*-flurbiprofen or ibuprofen was determined by SDS-PAGE using 50 μg of cell lysate and immunoblot analysis using a monoclonal antibody to human $p75^{NTR}$ and β-actin as the loading control.

constant level, it seems that *R*-flurbiprofen and ibuprofen inhibited survival through a mechanism other than COX-1 inhibition. DU-145 cells lacked any COX-2 expression, whereas PC-3 cells only exhibited expression upon *R*-flurbiprofen or ibuprofen treatment. An induction of COX-2 following NSAID treatment has been observed previously in PC-3 cells, as well as several other cell types (47–49). Although ibuprofen treatment led to an up-regulation of COX-2, the effect would be negated because ibuprofen would inhibit COX-2 activity. Because *R*-flurbiprofen lacks COX inhibitory activity, COX-2 could potentially remain active upon induction by *R*-flurbiprofen. However, 48-h *R*-flurbiprofen treatment of PC-3 cells was more potent for decreased survival than *R*-flurbiprofen in DU-145 cells, as well as more potent than ibuprofen treatment in both PC-3 and DU-145 cells. Therefore, there is no evidence that an induction of COX-2 in PC-3 cells promotes survival or reduces the effect of *R*-flurbiprofen treatment. Indeed, induction of COX-2 in PC-3 cells was associated with the greatest decrease in cell survival. This further supports the hypothesis that these drugs act as anticancer agents independently of COX-2.

$p75^{NTR}$ belongs to the TNFR superfamily, which includes Fas, $p55^{TNFR}$, DR3, DR4, DR5, and DR6 (35). These receptors initiate instructive apoptosis through a homologous intracellular death domain (50). With the exception of $p75^{NTR}$, they induce apoptosis upon ligand binding (51). In contrast, $p75^{NTR}$ initiates apoptosis and suppresses growth in a ligand-independent manner in prostate and bladder cancer cells (26–29). Previous studies show that some NSAIDs up-regulate expression of various death receptors (52, 53). To eliminate the possibility that the observed decrease in survival was due to apoptosis initiated by another member of the TNFR family, we examined expression of the remaining six family members in PC-3 cells following *R*-flurbiprofen or ibuprofen treatment. Neither $p55^{TNFR}$ nor DR6 were detected with or without treatment. Fas and DR5 were weakly expressed and remained constant upon treatment. DR3 expression was stronger than the other family members, but weak relative to $p75^{NTR}$, and remained constant following treatment. DR4 seems slightly up-regulated by ibuprofen; however, its expression levels were minimal relative to the robust induction of $p75^{NTR}$. Similar results were observed in the presence of the appropriate ligand for each receptor. Therefore, other than $p75^{NTR}$, the death receptors capable of initiating apoptosis do not seem to play a significant role in the decreased survival caused by *R*-flurbiprofen and ibuprofen treatment of PC-3 and DU-145 prostate cancer cells.

It seems that *R*-flurbiprofen and ibuprofen decrease survival of PC-3 and DU-145 cells by a COX-independent mechanism. $p75^{NTR}$ is the only TNFR superfamily member significantly up-regulated by treatment with aryl propionic acids, and its up-regulation is associated with decreased survival in both cell lines. This suggests $p75^{NTR}$ induction as a COX-independent mechanism initiating the observed decrease in survival. Hence, three different approaches including $p75^{NTR}$-targeting siRNA and two different $p75^{NTR}$ dominant negatives were used to examine a causal relationship between $p75^{NTR}$ levels and decreased cell survival. Each method increased survival of PC-3 and DU-145 cells treated with *R*-flurbiprofen or ibuprofen relative to untransfected cells. Collectively, these results provide strong evidence that $p75^{NTR}$ is at least partially causal of the decreased survival due to treatment with *R*-flurbiprofen or ibuprofen.

Because chemopreventive and chemotherapeutic drugs are often administered chronically, we examined whether long-term treatment enabled *R*-flurbiprofen and ibuprofen to maintain their anticancer activity at lower concentrations than observed with acute treatments (48 h). Both *R*-flurbiprofen and ibuprofen were highly effective in inducing $p75^{NTR}$ and reducing growth at lower concentrations after chronic treatment. In particular, long-term *R*-flurbiprofen treatment at the lowest concentration of 0.1 mmol/L exhibited an 85% reduction of growth relative to the control and essentially total loss of growth at concentrations of 0.3 mmol/L or greater in PC-3 cells. Likewise, in DU-145 cells, 0.1 mmol/L long-term *R*-flurbiprofen treatment resulted in 66% reduction of growth and almost a total loss of growth at 0.5 mmol/L treatment. This dramatic loss of growth also correlates with the induction of $p75^{NTR}$, which occurred in a dose-dependent manner starting at 0.1 mmol/L. Similarly, ibuprofen exhibited a substantial loss of growth due to chronic treatment at lower concentrations relative to acute 48-h treatment at higher concentrations in both PC-3 and DU-145 cells. The results of the chronic treatment experiments are especially significant because they indicate that these drugs, especially *R*-flurbiprofen, could be highly effective in inhibiting prostate cancer growth at clinically achievable concentrations with long-term use. In clinical trials, *R*-flurbiprofen and ibuprofen have been given at doses that result in plasma concentrations of 0.14 and 0.48 mmol/L, respectively (54). These concentrations are within the range of concentrations used in our chronic study. Hence, the data collectively demonstrate the activity of *R*-flurbiprofen and ibuprofen as anticancer agents in the prostate and convincingly implicate $p75^{NTR}$ induction as a COX-independent mechanism by which this anticancer activity is achieved.

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The p38 MAPK Pathway Mediates Aryl Propionic Acid–Induced Messenger RNA Stability of p75^{NTR} in Prostate Cancer Cells

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Abstract

The p75^{NTR} acts as a tumor suppressor in the prostate, but its expression is lost as prostate cancer progresses and is minimal in established prostate cancer cell lines such as PC-3, DU-145, and LNCaP. Previously, we showed that treatment with R-flurbiprofen or ibuprofen induced p75^{NTR} expression in PC-3 and DU-145 cells leading to p75^{NTR}-mediated decreased survival. Here, we investigate the mechanism by which these drugs induce p75^{NTR} expression. We show that the observed increase in p75^{NTR} protein due to R-flurbiprofen and ibuprofen treatment was accompanied by an increase in p75^{NTR} mRNA, and this increase in mRNA was the result of increased mRNA stability and not by an up-regulation of transcription. In addition, we show that treatment with R-flurbiprofen or ibuprofen led to sustained activation of the p38 mitogen-activated protein kinase (MAPK) pathway. Furthermore, inhibition of the p38 MAPK pathway with the p38 MAPK–specific inhibitor SB202190 or by small interfering RNA (siRNA) knockdown of p38 MAPK protein prevented induction of p75^{NTR} by R-flurbiprofen and ibuprofen. We also observed that siRNA knockdown of MAPK-activated protein kinase (MK)-2 and MK3, the kinases downstream of p38 MAPK that are responsible for the mRNA stabilizing effects of the p38 MAPK pathway, also prevented an induction of p75^{NTR} by R-flurbiprofen and ibuprofen. Finally, we identify the RNA stabilizing protein HuR and the posttranscriptional regulator eukaryotic translation initiation factor 4E as two possible mechanisms by which the p38 MAPK pathway may increase p75^{NTR} expression. Collectively, the data suggest that R-flurbiprofen and ibuprofen induce p75^{NTR} expression by increased mRNA stability that is mediated through the p38 MAPK pathway. [Cancer Res 2007;67(23):11402–10]

Introduction

The p75^{NTR} (neurotrophin receptor) is a member of the tumor necrosis factor (TNF) receptor superfamily that share a conserved intracellular death domain capable of inducing apoptosis and suppressing growth (1, 2). It binds neurotrophin ligands with similar affinity; however, unlike other members of the TNF receptor superfamily, it is able to induce apoptosis and suppress growth in the unbound state (3–8). p75^{NTR} has been shown to act as a tumor suppressor in the prostate (5, 7, 9). However, whereas normal prostate epithelial cells express high levels of p75^{NTR}, expression is lost as prostate cancer progresses (10). In addition, although the gene remains intact, expression is very low in metastatic prostate cancer cell lines PC-3, DU-145, and LNCaP (10). Exogenous reexpression of p75^{NTR} in PC-3 cells led to increased apoptosis, reduced proliferation, and a decreased ability of these cells to form tumors in mice, thus indicating potential for treatments that result in the reexpression of p75^{NTR} in prostate tumor cells (5, 7–9, 11).

Nonsteroidal anti-inflammatory drugs are typically used to relieve inflammation by inhibiting cyclooxygenase (COX) activity; however, many of these drugs seem to possess anticancer activity that is independent of their COX inhibitory activity (12). Flurbiprofen and ibuprofen are members of the aryl propionic acid class of nonsteroidal anti-inflammatory drugs, also referred to as profens, and have shown anticancer activity in the prostate. For example, treatment with the enantiomer R-flurbiprofen, which lacks COX inhibitory activity, was able to slow progression of prostate cancer in the transgenic adenocarcinoma of the mouse prostate mouse (13). In addition, long-term ibuprofen use has been associated with decreased prostate cancer risk, and treatment of prostate cancer cells with ibuprofen resulted in decreased survival (14–16). Recently, we showed that treatment of PC-3 and DU-145 prostate cancer cells with R-flurbiprofen or ibuprofen resulted in a strong induction of p75^{NTR}, which led to p75^{NTR}-mediated apoptosis and decreased survival (17). The results of this study were significant because they showed that p75^{NTR} expression is inducible, and that drugs which induce p75^{NTR} in prostate cancer cells have therapeutic potential.

The p38 mitogen-activated protein kinase (MAPK) pathway is activated in response to cell stresses and external stimuli such as heat, UV light, osmotic shock, and inflammatory cytokines (18). It mediates various cellular processes including apoptosis, senescence, inflammation, and tumorigenesis through decreased p38 MAPK activity, which has been associated with tumor progression (18–20). In addition, p38 MAPK has been strongly implicated as a regulator of mRNA stability (18, 21, 22). p38 MAPK is activated through phosphorylation by upstream kinases MAPK kinase kinase (MKK)-3 and MKK6 and, on activation, phosphorylates a number of downstream targets (18). Among these targets are the kinases MAPK-activated protein kinase (MK)-2 and MK3, which have been shown to be responsible for mediating the effects of the p38 MAPK pathway on mRNA stability (21, 23).

In this study, we investigated the mechanism by which the profens, R-flurbiprofen and ibuprofen, induce p75^{NTR} expression in PC-3, DU-145, and LNCaP prostate cancer cells. We showed that induction of p75^{NTR} protein expression corresponded to increased p75^{NTR} mRNA levels that were due to increased mRNA stability and not to an up-regulation of transcription. In addition, we showed that R-flurbiprofen and ibuprofen treatment led to sustained activation of the p38 MAPK pathway and that this pathway was necessary for induction of p75^{NTR} expression.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Cell lines, culture conditions, and reagents. PC-3 and DU-145 cells, the only two prostate tumor cell lines included in the NIH Developmental Therapeutics Program anticancer drug discovery program, and the androgen receptor–responsive LNCaP human prostate cancer cells were purchased from the tissue culture core facility of the Georgetown University Lombardi Comprehensive Cancer Center and maintained as previously described (17).

Ibuprofen (Sigma Chemical Co.) and R-flurbiprofen (Myriad Pharmaceuticals, Inc.) were dissolved in DMSO and used at final concentrations of 0, 0.25, 0.5, 1.0, or 2.0 mmol/L. Actinomycin D (Tocris Bioscience) and SB202190 (Calbiochem) were dissolved in DMSO and used at final concentrations of 5 μg/mL and 20 μmol/L, respectively.

Reverse transcription-PCR. RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Reverse transcription-PCR (RT-PCR) was done with the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen) using equal amounts of RNA according to the following program: 47°C for 30 min; 94°C for 2 min; 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; and 72°C for 5 min. Primers for p75^{NTR} and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were described previously (17).

Immunoblot analysis. Lysates were prepared as described previously (11). Cytoplasmic and nuclear fractions were prepared with a nuclear extract kit (Active Motif). SDS-PAGE and immunoblot analysis were done as described previously (17). The following primary antibodies were used: mouse monoclonal anti-p75^{NTR} (Millipore; 1:2,000); rabbit polyclonal antibodies to phosphorylated p38 MAPK, p38 MAPK, p38α, phosphorylated MK2, MK2, phosphorylated eukaryotic translation initiation factor 4E (eIF4E), and eIF4E (Cell Signaling Technology; 1:1,000); rabbit polyclonal anti-HuR (Millipore; 1:1,000); mouse monoclonal anti-p38β (Zymed Laboratories) and anti-MK3 (Affinity BioReagents; 2 μg/mL); mouse monoclonal anti-Hu protein (Abcam, Inc.; 1:1,000); mouse monoclonal anti–α-tubulin and rabbit polyclonal anti–lamin B1 (Abcam; 1:3,000); and mouse monoclonal anti–β-actin (Sigma; 1:5,000). Goat anti-mouse and goat anti-rabbit horseradish peroxidase–conjugated secondary antibodies (Bio-Rad Laboratories) were used at 1:3,000. Membranes used for detection of phosphorylated p38 MAPK, MK2, or eIF4E were subsequently stripped and reprobed for total p38 MAPK, MK2, or eIF4E, respectively.

Small interfering RNA transfection. Cells were transfected for 72 h with nontargeting small interfering RNA (siRNA) or siRNA specific for p38α (J-003512-20), p38β (J-003972-11), MK2 (J-003516-11), MK3 (J-005014-07), or HuR (J-003773-08; Dharmacon RNA Technologies) at final concentrations of 100 nmol/L according to the manufacturer's protocol. Transfection reagent DharmaFECT 1 was used for DU-145 cells and DharmaFECT 2 was used for PC-3 and LNCaP cells (Dharmacon).

Luciferase assay. PC-3, DU-145, and A875 cells were cotransfected with either the pGL3-2.1 kb p75^{NTR} promoter-luciferase construct (gift from Emil Bogenmann, Children's Hospital, Los Angeles, CA) or empty vector and the *Renilla* luciferase reporter vector phRL-TK (Promega) using GeneJammer transfection reagent (Stratagene) for 48 h. Following transfection, PC-3 and DU-145 cells were treated with 2 mmol/L R-flurbiprofen, 2 mmol/L ibuprofen, or DMSO vehicle control for 24 h, whereas A875 cells were treated with isotonic medium (320 mosm/L) or hypoosmotic medium (160 mosm/L) prepared by a 1:1 dilution of complete medium with water. Luciferase activity was measured using the Dual-Luciferase Reporter Assay (Promega) and normalized to the empty vector and *Renilla* luciferase activity.

Immunoprecipitation RT-PCR. Equal numbers of treated cells were collected, washed in cold PBS, and incubated on ice for 30 min in 1 mL of lysis buffer (1% Igepal Ca-630, 0.5% sodium deoxycholate, 0.1% SDS in PBS) containing 10 μg/mL Protease Inhibitor Cocktail (Sigma) and 100 units of RNaseOUT (Invitrogen) per sample. Samples were then centrifuged at 10,000 × *g* for 15 min at 4°C. Ten micrograms of HuR antibody (Millipore) were added to the supernatant of each sample, with the exception of the negative controls, and samples were rocked at 4°C for 30 min. Fifty microliters of protein A/G Plus-agarose beads (Santa Cruz Biotechnologies) were added to each sample, and samples were rocked at 4°C for 30 min. Samples were centrifuged at 2,000 × *g* for 2 min at 4°C, the supernatant was discarded, and the beads were washed eight times with 1 mL of lysis buffer. RNA was isolated from the beads using TRIzol reagent (Invitrogen) according to the manufacture's protocol. RT-PCR was done as described above for determination of p75^{NTR} mRNA level using equal volumes of RNA and 32 cycles of PCR.

Results

R-Flurbiprofen and ibuprofen increase p75^{NTR} mRNA level. To determine the mechanism by which the profens, R-flurbiprofen and ibuprofen, induce p75^{NTR} expression in PC-3 and DU-145 human prostate cancer cell lines, we first compared the levels of p75^{NTR} protein and mRNA following treatment with various concentrations of each profen (Fig. 1*A*). There was a strong correlation between p75^{NTR} protein and mRNA, indicating that increased mRNA levels are responsible for the induction of p75^{NTR} protein by R-flurbiprofen and ibuprofen. A strong increase in p75^{NTR} protein and mRNA levels was observed in PC-3 cells following treatment with 0.5 mmol/L R-flurbiprofen and 1.0 mmol/L ibuprofen, and in DU-145 cells with 1.0 mmol/L R-flurbiprofen and 2.0 mmol/L ibuprofen. Therefore, in subsequent experiments, we used 2.0 mmol/L R-flurbiprofen and ibuprofen because at this concentration, each of these profens induced high levels of p75^{NTR} in both cell types. The level of p75^{NTR} mRNA was also determined at various time points following profen treatment, and an induction was first observed within 2 to 4 h of treatment (Fig. 1*B*). We also compared levels of p75^{NTR} protein and mRNA in the androgen-responsive LNCaP cell line following treatment with several concentrations of ibuprofen, and again observed a strong correlation between p75^{NTR} protein and mRNA (Supplementary Fig. S1*A*). In addition, we observed a similar time course for induction of p75^{NTR} mRNA in LNCaP cells treated with ibuprofen (Supplementary Fig. S1*B*).

R-Flurbiprofen and ibuprofen increase p75^{NTR} mRNA stability. Because the amount of transcript in a cell is a function of the rate of transcription and the rate of degradation, the observed increase in p75^{NTR} mRNA in PC-3 and DU-145 cells following R-flurbiprofen and ibuprofen treatment must be due to either an up-regulation of transcriptional activity or a decrease in the rate of degradation of the p75^{NTR} transcript, or both. Thus, we examined transcriptional activity and mRNA stability following treatment with profens. PC-3 and DU-145 cells were transfected with a p75^{NTR} promoter-luciferase construct followed by treatment with R-flurbiprofen or ibuprofen, and promoter activity was compared with DMSO control–treated cells (Fig. 2*A*). Treatment with profens did not result in a significant change in p75^{NTR} promoter activity, suggesting that the increase in mRNA was not due to transcriptional up-regulation of the *p75^{NTR}* gene. Previously, p75^{NTR} promoter activity was shown to be up-regulated in the A875 melanoma cell line on exposure to hypoosmotic medium (24). Therefore, to show that the p75^{NTR} promoter-luciferase construct was functional, we transfected A875 cells with the construct and compared luciferase activity in cells exposed to isotonic versus hypoosmotic medium. Consistent with previously published data, treatment with hypoosmotic medium resulted in 17-fold increased p75^{NTR} promoter activity (Fig. 2*A*; ref. 24). Therefore, increased levels of p75^{NTR} mRNA and protein in PC-3 and DU-145 cells treated with R-flurbiprofen or ibuprofen were not a consequence of changed transcriptional activity.

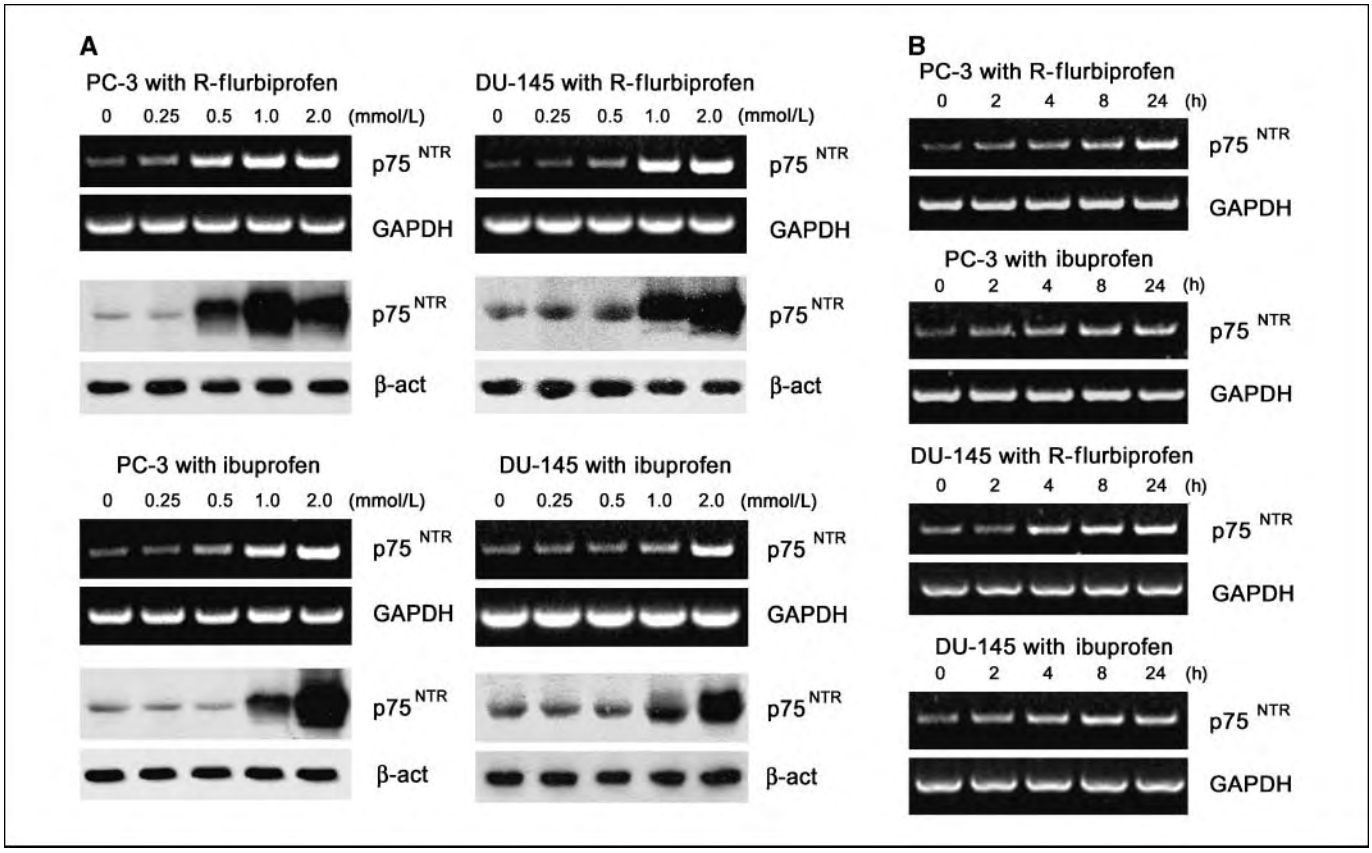

Figure 1. *A,* $p75^{NTR}$ mRNA and protein levels following R-flurbiprofen and ibuprofen treatment. PC-3 and DU-145 cells were treated with 0 to 2 mmol/L R-flurbiprofen or ibuprofen for 24 h. Cell lysates were collected and equal amounts of protein were subjected to SDS-PAGE and immunoblot analysis with an antibody to $p75^{NTR}$ or β-actin (*β-act*) for the loading control. Alternatively, RNA was isolated from PC-3 and DU-145 cells after 24 h of R-flurbiprofen or ibuprofen treatment, and $p75^{NTR}$ and GAPDH mRNA levels were determined by RT-PCR. *B,* time course induction of $p75^{NTR}$ mRNA levels. PC-3 and DU-145 cells were treated with 2 mmol/L R-flurbiprofen or ibuprofen for 0, 2, 4, 8, or 24 h. RNA was isolated at each time point and levels of $p75^{NTR}$ and GAPDH were determined by RT-PCR.

The stability of the $p75^{NTR}$ transcript was determined with the transcriptional inhibitor actinomycin D. PC-3 and DU-145 cells were treated with R-flurbiprofen, ibuprofen, or DMSO for 8 h followed by the addition of actinomycin D. $p75^{NTR}$ mRNA level was determined by RT-PCR at several time points following actinomycin D addition (Fig. 2*B* and *C*). The majority of $p75^{NTR}$ mRNA from DMSO control–treated PC-3 and DU-145 cells was degraded within 2 and 4 h, respectively. In contrast, $p75^{NTR}$ mRNA was still present at high levels in profen-treated cells 12 h following the addition of actinomycin D. Therefore, profen treatment strongly enhanced $p75^{NTR}$ mRNA stability but did not influence transcription, suggesting that increased mRNA stability is responsible for the induction of $p75^{NTR}$ expression by R-flurbiprofen and ibuprofen (Fig. 2). We also observed an increase in $p75^{NTR}$ mRNA stability in LNCaP cells treated with ibuprofen relative to DMSO control–treated cells (Supplementary Fig. S1*C* and *D*).

R-Flurbiprofen and ibuprofen activate the p38 MAPK pathway. The p38 MAPK pathway has been strongly implicated in the stabilization of several mRNAs through the downstream kinases MK2 and MK3, which are activated upon phosphorylation by p38 MAPK (21, 23). p38 MAPK is also activated by phosphorylation. Therefore, we determined the phosphorylation status of p38 MAPK and MK2 at several time points in PC-3 and DU-145 cells following treatment with R-flurbiprofen or ibuprofen and in LNCaP cells following treatment with ibuprofen (Fig. 3*A* and Supplementary Fig. S2*A*). In all cases, profen treatment led to sustained activation of the p38 MAPK pathway, which could be observed even 8 h after treatment of each cell line.

The p38 MAPK pathway is involved in the induction of $p75^{NTR}$ by R-flurbiprofen and ibuprofen. Increased $p75^{NTR}$ mRNA stability seems to be the mechanism by which R-flurbiprofen and ibuprofen induce $p75^{NTR}$ expression, and these drugs activate the p38 MAPK pathway, which is known to be involved in mRNA stabilization. Pretreatment of PC-3 and DU-145 cells with the p38 MAPK inhibitor SB202190 followed by treatment with R-flurbiprofen or ibuprofen prevented an induction of $p75^{NTR}$, indicating that p38 MAPK is involved in profen-mediated induction of $p75^{NTR}$ (Fig. 3*B*). To further confirm the role of the p38 MAPK pathway in profen-mediated induction of $p75^{NTR}$, we also used p38 MAPK siRNA to specifically knock down p38 MAPK protein. There are two ubiquitously expressed isoforms of p38 MAPK, p38α and p38β, which are capable of phosphorylating MK2 and MK3 (18, 23, 25). PC-3 and DU-145 cells were transfected with nontargeting siRNA, p38α siRNA, p38β siRNA, or siRNAs for p38α and p38β together. The efficacy and specificity of the siRNAs were determined by Western blot (Fig. 4*A*). Transfection of cells with p38α siRNA or p38α and p38β siRNAs together before profen treatment prevented an induction of $p75^{NTR}$ relative to untransfected cells or cells transfected with nontargeting siRNA (Fig. 4*B*). Transfection with p38β siRNA alone was less effective in preventing induction of $p75^{NTR}$ than transfection with p38α siRNA. Because the p38β isoform seems to be expressed at much lower

levels than p38α, it is not surprising that knockdown of p38β alone only modestly affected induction of p75^{NTR} because p38α was still present. Expression of p38α was also much greater than that of p38β in LNCaP cells (not shown), and consistently, induction of p75^{NTR} by ibuprofen was prevented in LNCaP cells transfected with p38α siRNA (Supplementary Fig. S2*C*).

Because the MK2 and MK3 kinases are downstream of p38 MAPK and have both been shown to mediate mRNA stabilization, we used siRNA for MK2 and MK3 to determine if they are involved in the induction of p75^{NTR} by R-flurbiprofen or ibuprofen. PC-3 and DU-145 cells were transfected with nontargeting siRNA, MK2 siRNA, MK3 siRNA, or siRNAs for MK2 and MK3 in combination. The efficacy and specificity of these siRNAs were tested in PC-3 and DU-145 cells by Western blot (Fig. 5*A*). Transfection with MK2 siRNA or MK3 siRNA separately before profen treatment resulted in decreased induction of p75^{NTR} (Fig. 5*B*). However, transfection with MK2 siRNA and MK3 siRNA together, resulting in the simultaneous knockdown of both proteins, was more effective in preventing induction of p75^{NTR} by R-flurbiprofen or ibuprofen than knockdown of MK2 or MK3 separately (Fig. 5*B*). This is consistent with previous observations that MK2 and MK3 are activated in parallel by p38 MAPK, have similar substrates, and are both strongly expressed in PC-3 and DU-145 cells (Fig. 5*A*; refs. 23, 25). Likewise, in LNCaP cells, simultaneous siRNA knockdown of MK2 and MK3 was successful in preventing induction of p75^{NTR} expression by ibuprofen (Supplementary Fig. S2*C*).

p75^{NTR} mRNA is a target of the RNA stabilizing protein HuR. The HuR is an RNA binding protein that enhances the stability of target mRNAs (26, 27). It has been shown that activation of the p38 MAPK pathway results in the translocation of HuR from the nucleus to the cytoplasm where it binds to the 3′-untranslated regions (UTR) of various mRNAs containing a core AUUUA sequence (20, 28–31). The p75^{NTR} transcript contains two AUUUA sites in the 3′-UTR, suggesting HuR binding as a possible mechanism by which R-flurbiprofen and ibuprofen may stabilize p75^{NTR} mRNA. R-Flurbiprofen– and ibuprofen-treated cells exhibited modestly increased levels of cytoplasmic HuR (Fig. 6*A*). In addition, siRNA knockdown of HuR protein before profen treatment of PC-3 and

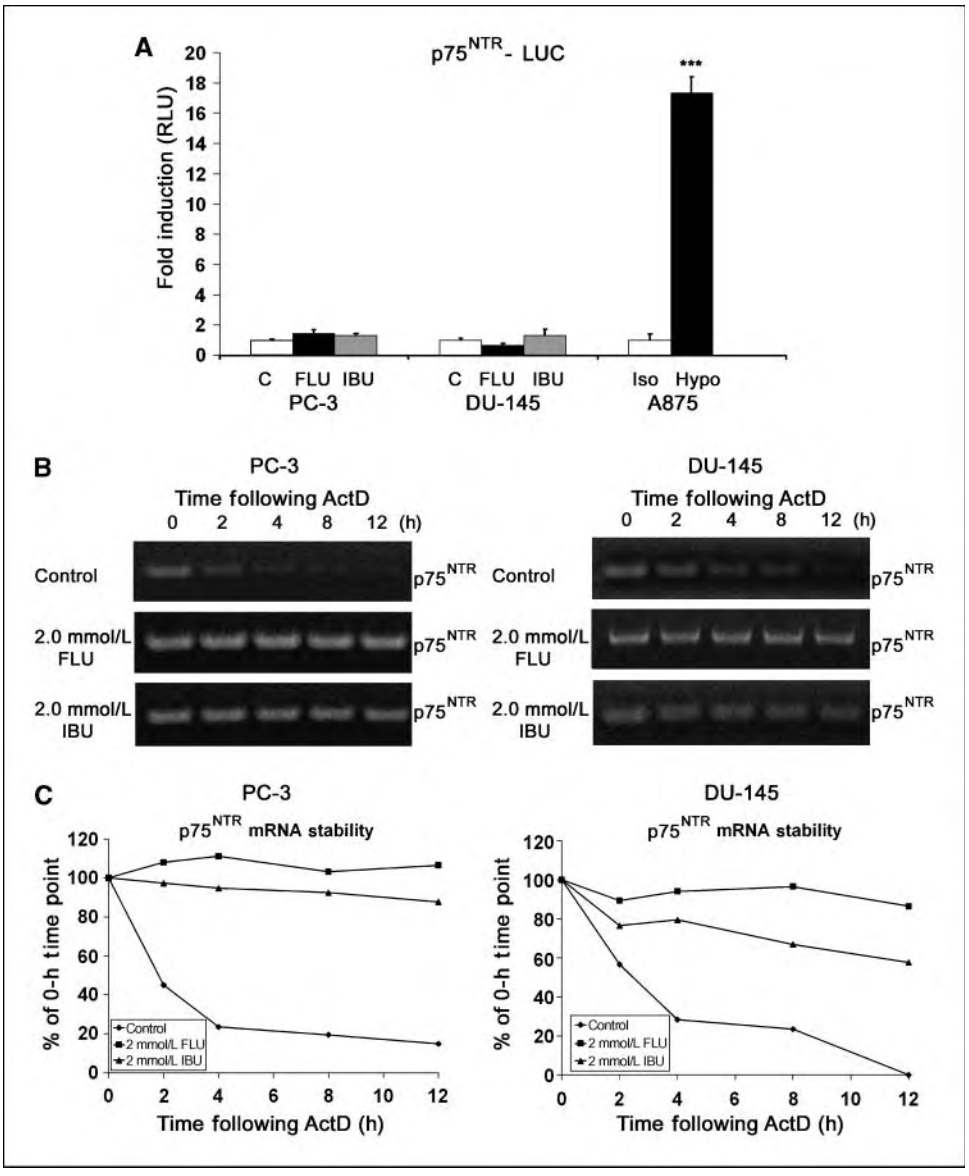

Figure 2. *A,* p75^{NTR} promoter activity following R-flurbiprofen and ibuprofen treatment. PC-3 and DU-145 cells were transfected for 48 h with a p75^{NTR} promoter-luciferase construct and then treated with 2 mmol/L R-flurbiprofen (*FLU*), 2 mmol/L ibuprofen (*IBU*), or DMSO vehicle control (*C*) for 24 h. Relative luciferase activity was determined after normalization to *Renilla* and graphed as fold induction relative to DMSO control–treated cells. As a positive control, A875 cells were transfected for 48 h with the p75^{NTR} promoter-luciferase construct and then exposed to isotonic (*Iso*) or hypoosmotic (*Hypo*) medium for 24 h. Fold induction of luciferase activity was determined for hypoosmotic treated cells relative to cells in isotonic medium. *Bars,* SE. ***, $P < 0.0001$ (ANOVA). *B* and *C,* increase in p75^{NTR} mRNA stability following R-flurbiprofen or ibuprofen treatment. PC-3 and DU-145 cells were treated with 2 mmol/L R-flurbiprofen, 2 mmol/L ibuprofen, or DMSO vehicle control for 8 h followed by the addition of 5 μg/mL actinomycin D (*ActD*). RNA was isolated at 0, 2, 4, 8, and 12 h following the addition of actinomycin D, and p75^{NTR} mRNA levels were determined by RT-PCR. The intensity of the bands for p75^{NTR} was analyzed by densitometry and graphed as a percentage of the 0-h time point.

DU-145 cells partially prevented induction of $p75^{NTR}$ relative to untransfected or nontargeting siRNA–transfected cells, suggesting that HuR is involved in the induction of $p75^{NTR}$ by R-flurbiprofen and ibuprofen (Fig. 6*B*). Similarly, transfection of LNCaP cells with HuR siRNA before ibuprofen treatment was effective in preventing increased $p75^{NTR}$ expression (Supplementary Fig. S2*C*). HuR belongs to the embryonic lethal abnormal vision family of proteins, which also includes HuB, HuC, and HuD, all RNA stabilizing proteins that are typically expressed in neurons (32). Whereas HuR expression was detected in PC-3 and DU-145 cells (Fig. 6*A*), expression of the other family members could not be detected (not shown), indicating that they most likely do not participate in profen-mediated induction of $p75^{NTR}$. A direct interaction between the HuR protein and the $p75^{NTR}$ transcript was shown by an immunoprecipitation RT-PCR assay. Lysates of profen-treated PC-3 and DU-145 cells were immunoprecipitated with, or without, an HuR antibody and RNA was isolated from the immunoprecipitated beads, followed by RT-PCR for $p75^{NTR}$ mRNA. PC-3 and DU-145 cells treated with R-flurbiprofen or ibuprofen showed a direct interaction between HuR and $p75^{NTR}$ mRNA, whereas DMSO control–treated cells did not (Fig. 6*C*). In addition, $p75^{NTR}$ mRNA was not detected in samples from cells treated with ibuprofen but not incubated with the HuR antibody, indicating that the $p75^{NTR}$ mRNA detected in the treated samples was not nonspecific (Fig. 6*C*).

R-Flurbiprofen and ibuprofen increase phosphorylation of eIF4E. Although HuR seems to bind to the $p75^{NTR}$ transcript upon profen treatment, the inhibition of $p75^{NTR}$ induction in the presence of a substantial HuR knockdown was modest. This result suggests that there are additional mechanisms involved in the induction of $p75^{NTR}$ by the p38 MAPK pathway. eIF4E is a downstream, indirect target of p38 MAPK and is involved in several mechanisms of posttranscriptional regulation including translation initiation through 5′-cap binding, mRNA nuclear export, and mRNA stability (33, 34). We examined eIF4E phosphorylation following profen treatment in untransfected, nontargeting siRNA–transfected, and p38α siRNA–transfected PC-3 and DU-145 cells. R-Flurbiprofen and ibuprofen treatment resulted in increased phosphorylated eIF4E levels in both cell types, and the increase was inhibited to varying degrees in the p38α siRNA–transfected cells (Fig. 6*D*). These results identify eIF4E phosphorylation as another mechanism through which the p38 MAPK pathway may regulate $p75^{NTR}$ expression.

Discussion

The $p75^{NTR}$ exhibits tumor suppressor activity in prostate cancer cells, which is mediated through an intracellular death domain capable of inducing apoptosis and suppressing growth (2, 5, 7, 9). However, $p75^{NTR}$ expression is progressively lost in organ confined prostate cancer and is minimal in established metastatic prostate cancer cell lines (10). Significantly, our recent observations that the profens, R-flurbiprofen and ibuprofen, induced reexpression of $p75^{NTR}$ protein levels causal of $p75^{NTR}$-dependent decreased survival of prostate cancer cells has stimulated an investigation

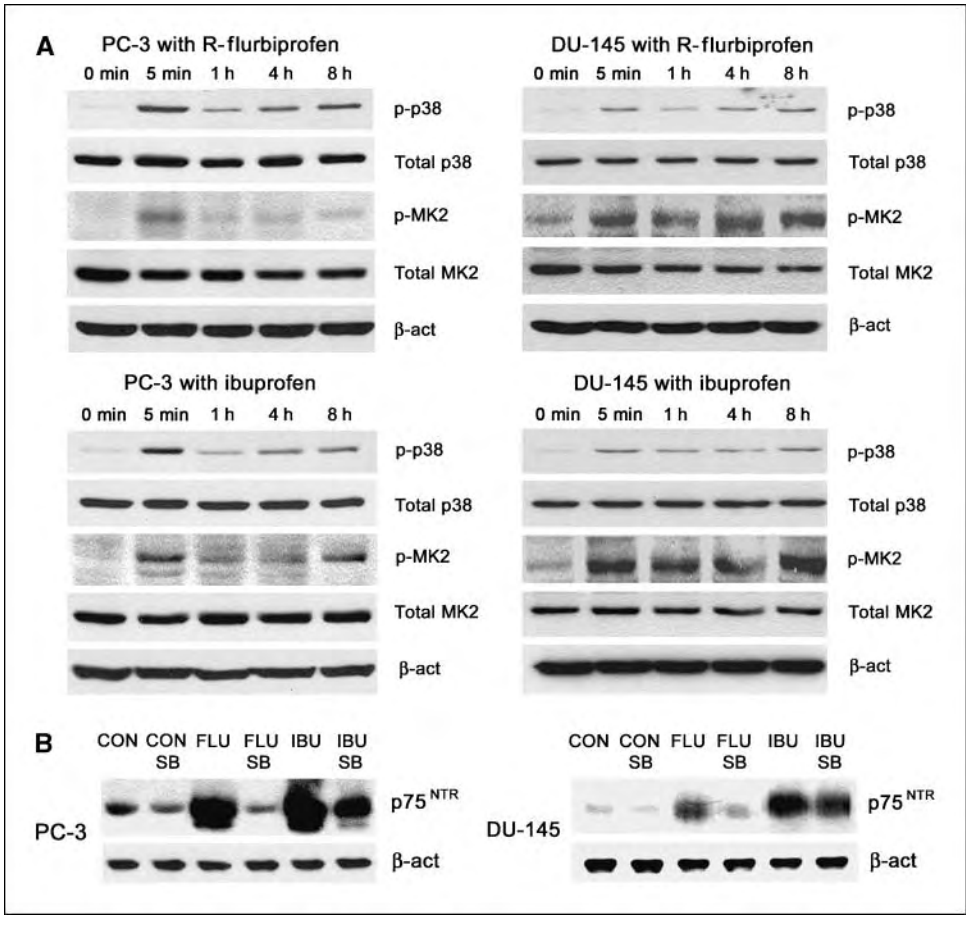

Figure 3. *A,* activation of the p38 MAPK pathway by R-flurbiprofen and ibuprofen. PC-3 and DU-145 cells were treated with 2 mmol/L R-flurbiprofen or 2 mmol/L ibuprofen for 0, 5 min, 1 h, 4 h, or 8 h. Cell lysates were collected and equal amounts of protein were subjected to SDS-PAGE and immunoblot analysis with antibodies to phosphorylated p38 MAPK (*p-p38*) or phosphorylated MK2 (*p-MK2*). Blots for phosphorylated p38 MAPK and phosphorylated MK2 were stripped and reprobed for total p38 MAPK and total MK2, respectively. β-actin was used as the loading control. *B,* the p38 MAPK inhibitor SB202190 prevents induction of $p75^{NTR}$ by R-flurbiprofen and ibuprofen. PC-3 and DU-145 cells were pretreated with or without 20 μmol/L SB202190 (*SB*) for 1 h and then treated with 2 mmol/L R-flurbiprofen, 2 mmol/L ibuprofen, or DMSO vehicle control (*CON*) for 24 h. Cell lysates were collected and equal amounts of protein were subjected to SDS-PAGE and immunoblot analysis with an antibody to $p75^{NTR}$ or β-actin for the loading control.

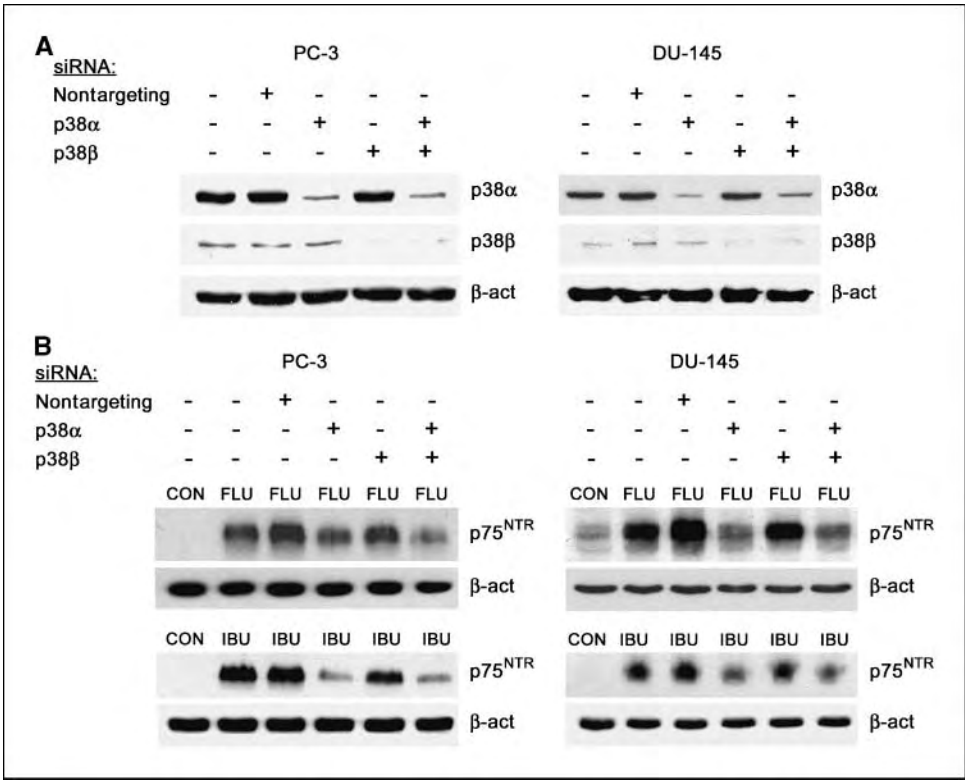

Figure 4. *A,* validation assay for siRNA knockdown of p38 MAPK. PC-3 and DU-145 cells were transfected for 72 h with nontargeting siRNA or siRNA targeting p38α, p38β, or siRNAs for p38α and p38β together. Cell lysates were collected and efficacy of siRNA knockdown was determined by SDS-PAGE and immunoblot analysis with antibodies to p38α and p38β. β-actin was used as the loading control. *B,* knockdown of p38 MAPK prevents induction of p75^{NTR} by R-flurbiprofen and ibuprofen. PC-3 and DU-145 cells were transfected with nontargeting siRNA, siRNA for p38α, siRNA for p38β, or siRNAs for p38α and p38β together for 72 h. Following transfection, cells were treated with 2 mmol/L R-flurbiprofen, 2 mmol/L ibuprofen, or DMSO vehicle control for 24 h. Cell lysates were collected and expression of p75^{NTR} was determined by SDS-PAGE and immunoblot analysis. β-actin was used as the loading control.

of the associated signal transduction cascade that mediates profen effects on prostate cancer cells (17). Increased p75^{NTR} protein levels in R-flurbiprofen– or ibuprofen-treated cells were accompanied by increased mRNA levels. This indicated that profen treatment influenced either the rate of mRNA degradation or the rate at which the gene was transcribed. Profen-treated cells showed no significant change in luciferase activity of the p75^{NTR} promoter construct relative to untreated cells. However, the transcriptional

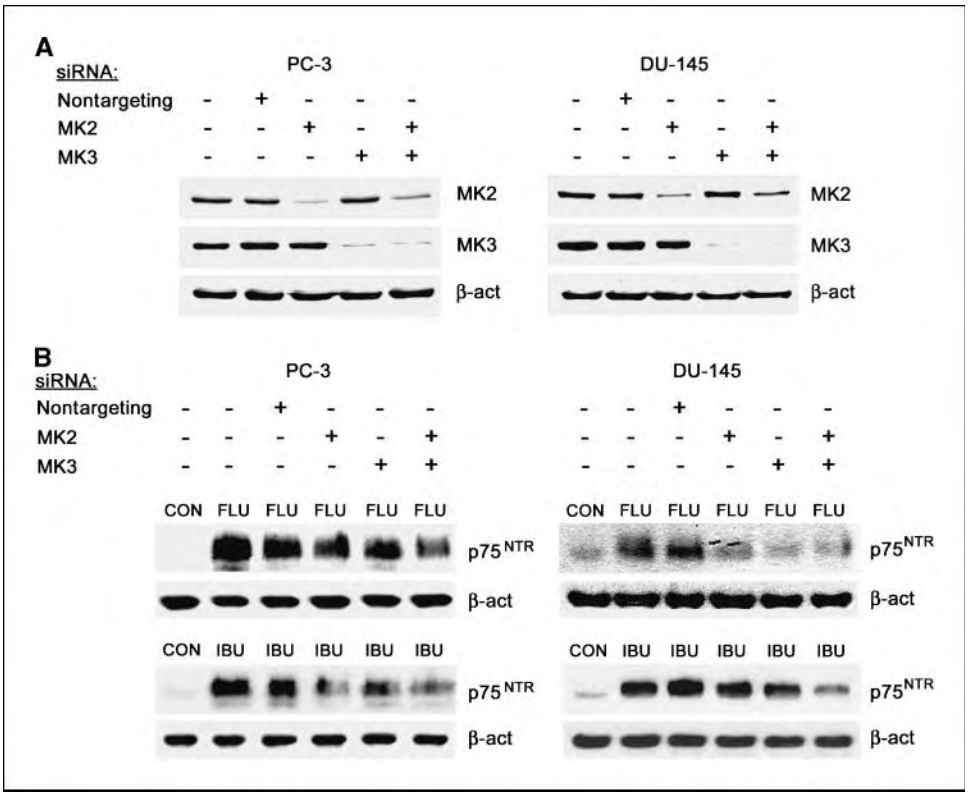

Figure 5. *A,* validation assay for siRNA knockdown of MK2 and MK3. PC-3 and DU-145 cells were transfected for 72 h with nontargeting siRNA, siRNA for MK2, siRNA for MK3, or siRNAs for MK2 and MK3 together. Cell lysates were collected and efficacy of siRNA knockdown was determined by SDS-PAGE and immunoblot analysis with antibodies to MK2 and MK3. β-actin was used as the loading control. *B,* knockdown of MK2 and MK3 prevents induction of p75^{NTR} by R-flurbiprofen and ibuprofen. PC-3 and DU-145 cells were transfected with nontargeting siRNA, siRNA for MK2, siRNA for MK3, or siRNAs for MK2 and MK3 together for 72 h. Following transfection, cells were treated with 2 mmol/L R-flurbiprofen, 2 mmol/L ibuprofen, or DMSO vehicle control for 24 h. Cell lysates were collected and expression of p75^{NTR} was determined by SDS-PAGE and immunoblot analysis. β-actin was used as the loading control.

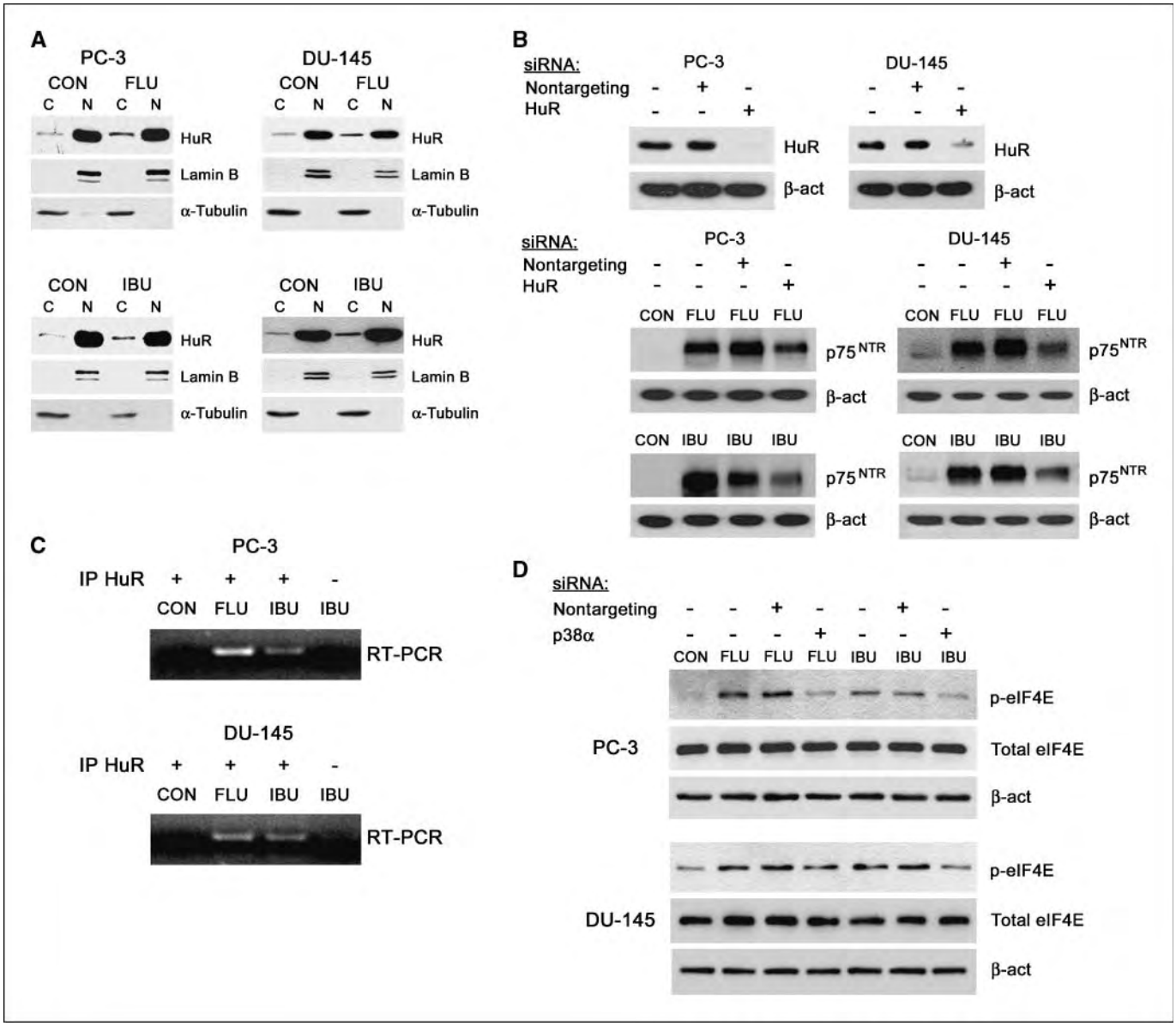

Figure 6. *A,* translocation of HuR from the nucleus to the cytoplasm following treatment with R-flurbiprofen or ibuprofen. PC-3 and DU-145 cells were treated for 2 h with 2 mmol/L R-flurbiprofen, 2 mmol/L ibuprofen, or DMSO vehicle control. Nuclear (*N*) and cytoplasmic (*C*) fractions were subjected to SDS-PAGE and immunoblot analysis with antibodies to HuR, lamin B1, and α-tubulin. *B,* knockdown of HuR prevents induction of $p75^{NTR}$ by R-flurbiprofen and ibuprofen. PC-3 and DU-145 cells were transfected for 72 h with nontargeting siRNA or siRNA targeting HuR. For the test for siRNA knockdown, cell lysates were collected and efficacy of siRNA knockdown was determined by SDS-PAGE and immunoblot analysis with an antibody to HuR or β-actin for the loading control. For examination of $p75^{NTR}$ expression, cells were treated with 2 mmol/L R-flurbiprofen, 2 mmol/L ibuprofen, or DMSO vehicle control for 24 h following siRNA transfection. Cell lysates were collected and expression of $p75^{NTR}$ was determined by SDS-PAGE and immunoblot analysis. β-actin was used as the loading control. *C,* HuR binds to the $p75^{NTR}$ transcript following treatment with R-flurbiprofen or ibuprofen. PC-3 and DU-145 cells were treated for 12 h with 2 mmol/L R-flurbiprofen, 2 mmol/L ibuprofen, or DMSO vehicle control. Cell lysates from equal numbers of cells were incubated with an antibody to HuR, with the exception of the negative controls, and then incubated with protein A/G Plus-agarose beads. Beads were washed, RNA was isolated from the beads, and $p75^{NTR}$ mRNA levels were determined by RT-PCR. *D,* R-flurbiprofen and ibuprofen increase phosphorylation of eIF4E through the p38 MAPK pathway. PC-3 and DU-145 cells were transfected for 72 h with nontargeting siRNA or p38α siRNA. Following transfection, cells were treated for 30 min with DMSO, 2 mmol/L R-flurbiprofen, or 2 mmol/L ibuprofen. Cell lysates were collected and phosphorylated eIF4E was detected by SDS-PAGE and immunoblot analysis. Blots for phosphorylated eIF4E were stripped and reprobed for total eIF4E. β-actin was used as the loading control.

inhibitor actinomycin D revealed a strong increase in $p75^{NTR}$ mRNA stability in cells treated with R-flurbiprofen or ibuprofen. Previously, our investigation of the mechanism by which several prostate cancer cell lines lose expression of $p75^{NTR}$ showed that although $p75^{NTR}$ is actively transcribed at levels comparable to the high-expressing A875 melanoma cell line, the prostate cancer cells contain <1% of the $p75^{NTR}$ mRNA found in A875 cells (10). Furthermore, transfection of prostate cancer cells with full-length $p75^{NTR}$ cDNA led to very modest $p75^{NTR}$ protein expression, whereas transfection with $p75^{NTR}$ cDNA lacking most of the 3′-UTR resulted in high protein expression. This suggested that although $p75^{NTR}$ is transcribed at a high level, prostate cancer cells have very little mRNA or protein due to increased mRNA instability that is mediated through the 3′-UTR. Interestingly, the $p75^{NTR}$ 3′-UTR is

~2 kb long, which is about four times the length of the average human 3′-UTR (35). It has been suggested that increased 3′-UTR length provides increased potential for posttranscriptional regulation through the 3′-UTR (35). Therefore, it seems likely that R-flurbiprofen and ibuprofen are able to induce p75^{NTR} expression by modulating the stability of the transcript and that mRNA stability is an important determinant in regulating the expression level of p75^{NTR} protein.

Because induction of p75^{NTR} by R-flurbiprofen and ibuprofen treatment seemed to be largely due to increased mRNA stability, we examined activity of the p38 MAPK pathway, which is considered an important regulator of mRNA stability (18, 21, 22). p38 MAPK is activated by phosphorylation, and R-flurbiprofen and ibuprofen caused increased p38 MAPK phosphorylation within 5 min of treatment. Increased activation was still noticeable 8 h following treatment. Because pretreatment with the p38 MAPK selective inhibitor SB202190 or siRNA knockdown of p38 MAPK before profen treatment prevented an induction of p75^{NTR}, it seems that the p38 MAPK pathway was involved in regulating profen-mediated induction of p75^{NTR}. The data suggest that activation of the p38 MAPK pathway is the primary mechanism responsible for increased p75^{NTR} expression. However, inhibition of p38 MAPK did not always result in complete inhibition of p75^{NTR} induction, and this may be due to participation of additional pathways. R-Flurbiprofen and ibuprofen induced activation of the kinase MK2, which is directly downstream of p38 MAPK. MK2 and the closely related MK3 are known to be responsible for mediating the mRNA stabilizing effects of the p38 MAPK pathway (22, 23). They are activated with similar kinetics, are able to compensate for one another, have similar substrates, and lead to stabilization of the same group of transcripts in response to lipopolysaccharide (LPS; refs. 23, 25). Not surprisingly, siRNA knockdown of MK2 and MK3 together was more effective in preventing induction of p75^{NTR} by R-flurbiprofen or ibuprofen than knockdown of either MK2 or MK3 separately, indicating that p38 MAPK is able to induce p75^{NTR} by acting through both MK2 and MK3.

Activation of the p38 MAPK pathway seems to be responsible for R-flurbiprofen– and ibuprofen-mediated induction of p75^{NTR} in prostate cancer cells. Significantly, there is an accumulating body of evidence linking p38 MAPK to tumor suppression. For example, inactivation of p38 MAPK, inactivation of the p38 MAPK activating kinases MKK3 and MKK6, and overexpression of the p38 MAPK phosphatase Wip1 are associated with increased tumorigenesis (36, 37). In addition, Wip1 was found to be amplified in primary breast tumors (36, 37). The tumor suppressor p53 is a direct substrate of p38 MAPK and is therefore one way in which p38 MAPK exerts its tumor-suppressing effects (18, 21, 36). However, p53 is inactivated by mutation in roughly half of all cancers (38). This is also the case for PC-3 cells, which are p53-null, and for DU-145 cells, which express mutated p53 (39). Here, we identify induction of p75^{NTR} expression as a novel mechanism by which p38 MAPK may achieve tumor suppressor activity. This may be applicable to other cancer types in addition to prostate cancer. For example, we previously showed that ibuprofen up-regulates p75^{NTR} in bladder cancer cells, which resulted in p75^{NTR}-mediated apoptosis (40). In addition, basic fibroblast growth factor treatment resulted in the p38 MAPK–dependent death of Ewing's sarcoma family of tumor cells and was associated with induction of p75^{NTR} that was prevented in the presence of a p38 MAPK selective inhibitor (41). Interestingly, p38 MAPK and p75^{NTR} have many overlapping roles in addition to induction of apoptosis and tumor suppression. For example, both are involved in the regulation of inflammation as well as response to brain injury (18, 24, 42–45). Therefore, it is possible that p38 MAPK achieves these roles, in part, by increasing expression of p75^{NTR}. Consistent with this hypothesis is the observation that LPS, which leads to the stabilization of several transcripts through the p38 MAPK/MK2 pathway, also induces p75^{NTR} expression (23, 46, 47).

One mechanism by which the p38 MAPK pathway has been shown to stabilize target transcripts involves the RNA binding protein HuR (22, 29–31). HuR is the only RNA binding protein repeatedly shown to stabilize transcripts containing the AUUUA sequence (22, 31). Its ability to stabilize target mRNAs is linked to its subcellular localization, and activation of p38 MAPK and MK2 has been shown to cause translocation of HuR from the nucleus to the cytoplasm, resulting in increased mRNA stability of a number p38 MAPK regulated genes (22, 29–31). The human p75^{NTR} transcript contains AUUUA sites located in the 3′-UTR at positions 2,946 and 3,124, and these are conserved in rat and mouse, suggesting that they may be involved in the regulation of p75^{NTR} expression. We showed that treatment with R-flurbiprofen or ibuprofen resulted in the binding of HuR to the p75^{NTR} transcript, and a modest increase in the cytoplasmic level of HuR was observed following profen treatment. However, siRNA knockdown of HuR before profen treatment only partially prevented an induction of p75^{NTR}. These data suggest that binding of HuR to the p75^{NTR} transcript is not the sole mechanism responsible for increased p75^{NTR} expression. This is not surprising given the elaborate picture of mRNA regulation that is emerging. Recent data suggest that posttrascriptional regulation of gene expression occurs through a plethora of RNA binding proteins that control the splicing, nuclear export, stability, localization, translation, and degradation of transcripts, often through specific sequential or structural elements located in the untranslated regions (48, 49). Therefore, given the exceptionally long 3′-UTR of the p75^{NTR} transcript, it is probable that other RNA binding proteins regulate expression as well, and this area requires further investigation. To provide insight into additional mechanisms through which the p38 MAPK pathway may influence p75^{NTR} mRNA stability, we examined phosphorylation of eIF4E. eIF4E is phosphorylated by kinases downstream of p38 MAPK (33). It is most well known as a 5′-cap binding protein involved in translation initiation. However, recent data suggest that it regulates gene expression at several levels (34). Interestingly, an increase in eIF4E activity is not associated with an increase in global translation, but rather translation of only a subset of transcripts (34). In addition, eIF4E has been shown to control the nuclear export of a different subset of transcripts (34). Finally, eIF4E is linked to control of mRNA stability in that removal of the 5′-cap is a key step in mRNA degradation, and competition between eIF4E and decapping enzymes has been shown (50). We show that profen treatment increases the level of phosphorylated eIF4E, and this seems to occur, at least partially, through the p38 MAPK pathway because the increase in phosphorylation is substantially inhibited in the presence of p38 MAPK siRNA. Therefore, these data identify modulation of eIF4E activity as another mechanism by which the p38 MAPK pathway may control posttranscriptional events in response to profen treatment.

In this study, we show for the first time that R-flurbiprofen and ibuprofen activate the p38 MAPK pathway in prostate cancer cells and that this pathway is necessary for induction of the p75^{NTR} tumor suppressor by these drugs. This mechanism of induction occurs in both androgen-independent PC-3 and DU-145 cells as

well as androgen-responsive LNCaP cells. Retrospective epidemiologic studies have shown that certain nonsteroidal anti-inflammatory drugs, including ibuprofen, reduce the incidence of prostate cancer (15, 16). Numerous recent studies have provided compelling evidence that inhibition of cancer cell growth by nonsteroidal anti-inflammatory drugs can occur through COX-independent mechanisms that have yet to be fully elucidated (12, 13, 17, 40). The enantiomer R-flurbiprofen lacks COX inhibitory activity, indicating that the activation of p38 MAPK and subsequent induction of $p75^{NTR}$ is a COX-independent mechanism by which these profens can achieve anticancer activity in the prostate (13). Because p38 MAPK is activated within 5 min of profen treatment, it is likely that R-flurbiprofen and ibuprofen are interacting with signaling components proximal to p38 MAPK. Further delineation of this mechanism may identify novel therapeutic targets and facilitate the discovery of more effective compounds that are able to induce $p75^{NTR}$ expression at lower concentrations.

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